Docket No.: PF-0417-2 DIV

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By: Emma Durrell
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Bandman et al.

Title: VESICLE TRAFFICKING PROTEINS

Serial No.:	09/556,178	Filing Date:	April 20, 2000
Examiner:	Strzelecka, T.	Group Art Unit:	1656

Box Fee Amendment
Commissioner for Patents
Washington, D.C. 20231

DECLARATION OF LARS MICHAEL FURNESS
UNDER 37 C.F.R. § 1.132

I, L. MICHAEL FURNESS, a citizen of the United Kingdom, residing at 2 Brookside, Exning, Newmarket, United Kingdom, declare that:

1. I was employed by Incyte Genomics, Inc. (hereinafter "Incyte") as a Director of Pharmacogenomics until December 31, 2001. I am currently under contract to be a Consultant to Incyte Genomics, Inc.

2. In 1984, I received a B.Sc.(Hons) in Biomolecular Science (Biophysics and Biochemistry) from Portsmouth Polytechnic.

From 1985-1987 I was at the School of Pharmacy in London, United Kingdom, during which time I analyzed lipid methyltransferase enzymes using a variety of protein analysis methods, including one-dimensional (1D) and two-dimensional (2D) gel electrophoresis, HPLC, and a variety of enzymatic assay systems.

I then worked in the Protein Structure group at the National Institute for Medical Research until 1989, setting up core facilities for nucleic acid synthesis and sequencing, as well as assisting in programs on protein kinase C inhibitors.

After a year at Perkin Elmer-Applied Biosystems as a technical specialist, I worked at the Imperial Cancer Research Fund between 1990-1992, on a Eureka-funded program collaborating with Amersham Pharmacia in the United Kingdom and CEPH (Centre d'Etude du Polymorphisme Humaine) in Paris, France, to develop novel nucleic acid purification and characterization methods.

In 1992, I moved to Pfizer Central Research in the United Kingdom, where I stayed until 1998, initially setting up core DNA sequencing and then a DNA arraying facility for gene expression analysis in 1993. My work also included bioinformatics and I was responsible for the support of all Pfizer neuroscience programs in the United Kingdom. This then led me into carrying out detailed bioinformatics and wet lab work on the sodium channels, including antibody generation, western and northern analyses, PCR, tissue distribution studies, and sequence analyses on novel sequences identified.

In1998 I moved to Incyte Genomics, Inc., in the Pharmacogenomics group, looking at the application of genomics and proteomics to the pharmaceutical industry. In 1999 I was appointed director of the LifeExpress Lead Program which used microarray and protein expression data to identify pharmacologically and toxicologically relevant mechanisms to assist in improved drug design and development.

On December 12, 2001 I founded Nuomics Consulting Ltd., in Exning, U.K., and I am currently employed as Managing Director. Nuomics Consulting Ltd. will be providing expert technical knowledge and advice to businesses around the areas of genomics, proteomics, pharmacogenomics, toxicogenomics and chemogenomics.

3. I have reviewed the specification of a United States patent application that I understand was filed on April 20, 2000 in the names of Olga Bandman et al. and was assigned Serial No. 09/556,178 (hereinafter "the Bandman '178 application"). Furthermore, I understand that this United States patent application was a divisional application of and claimed priority to United States patent application Serial No. 09/368,408 filed on August 4, 1999, which was itself a divisional application of and claimed priority to United States patent application Serial No. 08/967,364 filed on November 7, 1997 (hereinafter "the Bandman '364 application") having essentially the identical specification, with the exception of corrected typographical errors and reformatting. Thus page and line numbers may not match as between the Bandman '178 application and the Bandman '364 application. My remarks herein will therefore be directed to the Bandman '364 patent application, and November 7, 1997, as the relevant date of filing. In broad

overview, the Bandman '364 specification pertains to certain nucleotide and amino acid sequences and their use in a number of applications, including gene and protein expression monitoring applications that are useful in connection with (a) developing drugs (e.g., for the treatment of cancer), and (b) monitoring the activity of drugs for purposes relating to evaluating their efficacy and toxicity.

4. I understand that (a) the Bandman '178 application contains claims that are directed to an isolated polypeptide having the sequence shown as SEQ ID NO:1 (hereinafter "the SEQ ID NO:1 polypeptide"), and (b) the Patent Examiner has rejected those claims on the grounds that the specification of the Bandman '178 application does not disclose a substantial, specific and credible utility for the claimed SEQ ID NO:1 polypeptide. I further understand that whether or not a patent specification discloses a substantial, specific and credible utility for its claimed subject matter is properly determined from the perspective of a person skilled in the art to which the specification pertains at the time of the patent application was filed. In addition, I understand that a substantial, specific and credible utility under the patent laws must be a "real-world" utility.

5. I have been asked (a) to consider with a view to reaching a conclusion (or conclusions) as to whether or not I agree with the Patent Examiner's position that the Bandman '178 application and its parent, the Bandman '364 application, do not disclose a substantial, specific and credible "real-world" utility for the claimed SEQ ID NO:1 polypeptide, and (b) to state and explain the bases for any conclusions I reach. I have been informed that, in connection with my considerations, I should determine whether or not a person skilled in the art to which the Bandman '364 application pertains on November 7, 1997, would have concluded that the Bandman '364 application disclosed, for the benefit of the public, a specific beneficial use of the SEQ ID NO:1 polypeptide in its then available and disclosed form. I have also been informed that, with respect to the "real-world" utility requirement, the Patent and Trademark Office instructs its Patent Examiners in Section 2107.01 of the Manual of Patent Examining Procedure, 8th Edition, August 2001, under the heading I. Specific and Substantial Requirements, Research Tools:

> Many research tools such as gas chromatographs, screening assays, and nucleotide sequencing techniques have a clear, specific and unquestionable utility (e.g., they are useful in analyzing compounds). An assessment that focuses on whether an invention is useful only in a research setting thus does not address whether the specific invention is in fact "useful" in a patent sense. Instead, Office personnel

must distinguish between inventions that have a specifically identified substantial utility and inventions whose asserted utility requires further research to identify or reasonably confirm.

6. I have considered the matters set forth in paragraph 5 of this Declaration and have concluded that, contrary to the position I understand the Patent Examiner has taken, the specification of the Bandman '364 patent application disclosed to a person skilled in the art at the time of its filing a number of substantial, specific and credible real-world utilities for the claimed SEQ ID NO:1 polypeptide. More specifically, persons skilled in the art on November 7, 1997 would have understood the Bandman '364 application to disclose the use of the SEQ ID NO:1 polypeptide as a research tool in a number of gene and protein expression monitoring applications that were well-known at that time to be useful in connection with the development of drugs and the monitoring of the activity of such drugs. I explain the bases for reaching my conclusion in this regard in paragraphs 7-13 below.

7. In reaching the conclusion stated in paragraph 6 of this Declaration, I considered (a) the specification of the Bandman '364 application, and (b) a number of published articles and patent documents that evidence gene and protein expression monitoring techniques that were well-known before the November 7, 1997 filing date of the Bandman '364 application. The published articles and patent documents I considered are:

(a) Anderson, N.L., Esquer-Blasco, R., Hofmann, J.-P., Anderson, N.G., A Two-Dimensional Gel Database of Rat Liver Proteins Useful in Gene Regulation and Drug Effects Studies, Electrophoresis, 12, 907-930 (1991) (hereinafter "the Anderson 1991 article") (copy annexed at Tab A);

(b) Anderson, N.L., Esquer-Blasco, R., Hofmann, J.-P., Mehues, L., Raymackers, J., Steiner, S. Witzmann, F., Anderson, N.G., An Updated Two-Dimensional Gel Database of Rat Liver Proteins Useful in Gene Regulation and Drug Effect Studies, Electrophoresis, 16, 1977-1981 (1995) (hereinafter "the Anderson 1995 article") (copy annexed at Tab B);

(c) Wilkins, M.R., Sanchez, J.-C., Gooley, A.A., Appel, R.D., Humphery-Smith, I., Hochstrasser, D.F., Williams, K.L., Progress with Proteome Projects: Why all Proteins Expressed by a Genome Should be Identified and How To Do It, Biotechnology and

Genetic Engineering Reviews, 13, 19-50 (1995) (hereinafter "the Wilkins article") (copy annexed at Tab C);

(d) Celis, J.E., Rasmussen, H.H., Leffers, H., Madsen, P., Honore, B., Gesser, B., Dejgaard, K., Vandekerckhove, J., Human Cellular Protein Patterns and their Link to Genome DNA Sequence Data: Usefulness of Two-Dimensional Gel Electrophoresis and Microsequencing, FASEB Journal, 5, 2200-2208 (1991) (hereinafter "the Celis article") (copy annexed at Tab D);

(e) Franzen, B., Linder, S., Okuzawa, K., Kato, H., Auer, G., Nonenzymatic Extraction of Cells from Clinical Tumor Material for Analysis of Gene Expression by Two-Dimensional Polyacrylamide Gel Electrophoresis, Electrophoresis, 14, 1045-1053 (1993) (hereinafter "the Franzen article") (copy annexed at Tab E);

(f) Bjellqvist, B., Basse, B., Olsen, E., Celis, J.E., Reference Points for Comparisons of Two-Dimensional Maps of Proteins from Different Human Cell Types Defined in a pH Scale Where Isoelectric Points Correlate with Polypeptide Compositions, Electrophoresis, 15, 529-539 (1994) (hereinafter "the Bjellqvist article") (copy annexed at Tab F);

(g) Large Scale Biology Company Info; LSB and LSP Information; from http://www.lsbc.com (2001) (copy annexed at Tab G); and

(h) Pevsner, J., Hsu, S.-C., Hyde, P.S., and Scheller, R.H., Mammalian homologues of yeast vacuolar protein sorting (*vps*) genes implicated in Golgi-to-lysosome trafficking, Gene, 183, 7-14 (1996) (hereinafter "the Pevsner article") (copy annexed at Tab H).

8. Many of the published articles I considered (i.e., at least items (a)-(f) identified in paragraph 7) relate to the development of protein two-dimensional gel electrophoretic techniques for use in gene expression monitoring applications in drug development and toxicology. As I will discuss below, a person skilled in the art who read the Bandman '364 application on November 7, 1997 would have understood that application to disclose the SEQ ID NO:1 polypeptide to be useful for a number of gene and protein expression monitoring applications, e.g., in the use of two-dimensional polyacrylamide gel electrophoresis and western blot analysis of tissue samples in drug development and in toxicity testing.

9. Turning more specifically to the Bandman '364 specification, the SEQ ID NO:1 polypeptide is shown at pages 57-59 as one of nine sequences under the heading "Sequence Listing." The Bandman '364 specification specifically teaches that the "invention features a

substantially purified human vesicle trafficking protein (VTP) comprising an amino acid sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3, and SEQ ID NO:5." (Bandman '364 application at page 2, lines 23-25.). It further teaches that (a) the identity of the SEQ ID NO:1 polypeptide was determined from a "THP-1 cell line cDNA library (THP1PEB01)," (b) the SEQ ID NO:1 polypeptide is the vesicle trafficking protein referred to as "VTP-1" and is encoded by SEQ ID NO:2, and (c) northern analysis shows "the expression of VTP-1 in various cDNA libraries, at least 42% of which are immortalized or cancerous, at least 24% of which involve immune response, and at least 29% are expressed in fetal/infant tissues or organs" and therefore VTP-1 appears to play a role in inflammation and disorders associated with cell proliferation and apoptosis. (Bandman '364 application at p. 14, lines 5-29.)

The Bandman '364 application discusses a number of uses of the SEQ ID NO:1 polypeptide in addition to its use in gene expression monitoring applications. I have not fully evaluated these additional uses in connection with the preparation of this Declaration and do not express any views in this Declaration regarding whether or not the Bandman '364 specification discloses these additional uses to be substantial, specific and credible real-world utilities of the SEQ ID NO:1 polypeptide. Consequently, my discussion in this Declaration concerning the Bandman '364 application focuses on the portions of the application that relate to the use of the SEQ ID NO:1 polypeptide in gene and protein expression monitoring applications.

10. The Bandman '364 application discloses that the polynucleotide sequences disclosed therein, including the polynucleotides encoding the SEQ ID NO:1 polypeptide, are useful as probes in chip based technologies. They further teach that the chip based technologies can be used "for the detection and/or quantification of nucleic acid or protein" (Bandman '364 application at p. 25, lines 21-23).

The Bandman '364 application also discloses that the SEQ ID NO:1 polypeptide is useful in other protein expression detection technologies. The Bandman '364 application states that "[a] variety of protocols for detecting and measuring the expression of VTP, using either polyclonal or monoclonal antibodies specific for the protein are known in the art. Examples include enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA), and fluorescence activated cell sorting (FACS)." (Bandman '364 application at page 25, line 29 through page 26, line 2.) Furthermore, the Bandman '364 application discloses that "[a] variety of protocols including ELISA, RIA, and FACS for measuring VTP are known in the art and provide a basis for diagnosing altered or abnormal levels of VTP expression. Normal or standard values for VTP expression are

established by combining body fluids or cell extracts taken from normal mammalian subjects, preferably human, with antibody to VTP under conditions suitable for complex formation." (Bandman '364 application at page 38, lines 21-25.)

In addition, at the time of filing the Bandman '364 application, it was well known in the art that "gene" and protein expression analyses also included two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) technologies, which were developed during the 1980s, and as exemplified by the Anderson 1991 and 1995 articles (Tab A and Tab B). The Anderson 1991 article teaches that a 2-D PAGE map has been used to connect and compare hundreds of 2-D gels of rat liver samples from a variety of studies including regulation of protein expression by various drugs and toxic agents (Tab A at p. 907). The Anderson 1991 article teaches an empirically-determined standard curve fitted to a series of identified proteins based upon amino acid chain length (Tab A at p. 911) and how that standard curve can be used in protein expression analysis. The Anderson 1991 article teaches that "there is a long-term need for a comprehensive database of liver proteins" (Tab A at p. 912).

The Wilkins article is one of a number of documents that were published prior to the November 7, 1997 filing date of the Bandman '364 application that describes the use of the 2-D PAGE technology in a wide range of gene and protein expression monitoring applications, including monitoring and analyzing protein expression patterns in human cancer, human serum plasma proteins, and in rodent liver following exposure to toxins. In view of the Bandman '364 application, the Wilkins article, and other related pre-November 7, 1997 publications, persons skilled in the art on November 7, 1997 clearly would have understood the Bandman '364 application to disclose the SEQ ID NO:1 polypeptide to be useful in 2-D PAGE analyses for the development of new drugs and monitoring the activities of drugs for such purposes as evaluating their efficacy and toxicity, as explained more fully in paragraph 12 below.

With specific reference to toxicity evaluations, those of skill in the art who were working on drug development in November 7, 1997 (and for many years prior to November 7, 1997) without any doubt appreciated that the toxicity (or lack of toxicity) of any proposed drug they were working on was one of the most important criteria to be considered and evaluated in connection with the development of the drug. They would have understood at that time that good drugs are not only potent, they are specific. This means that they have strong effects on a specific biological target and minimal effects on all other biological targets. Ascertaining that a candidate drug affects its intended target, and identification of undesirable secondary effects (i.e., toxic side effects), had been for many years among the main challenges in developing new drugs. The ability

to determine which genes are positively affected by a given drug, coupled with the ability to quickly and at the earliest time possible in the drug development process identify drugs that are likely to be toxic because of their undesirable secondary effects, have enormous value in improving the efficiency of the drug discovery process, and are an important and essential part of the development of any new drug. In fact, the desire to identify and understand toxicological effects using the experimental assays described above led Dr Leigh Anderson to found the Large Scale Biology Corporation in 1985, in order to pursue commercial development of the 2-D electrophoretic protein mapping technology he had developed. In addition, the company focused on toxicological effects on the proteome as clearly demonstrated by its goals and by its senior management credentials described in company documents (see Tab G at pp. 1, 3, and 5).

Accordingly, the teachings in the Bandman '364 application, in particular regarding use of SEQ ID NO:1 in differential gene and protein expression analysis (2-D PAGE maps) and in the development and the monitoring of the activities of drugs, clearly includes toxicity studies and persons skilled in the art who read the Bandman '364 application on November 7, 1997 would have understood that to be so.

11. As previously discussed (*supra*, paragraphs 7 and 8), my experience with protein analysis methods in the mid-1980s and the several publications annexed to this Declaration at Tabs A through F evidence information that was available to the public regarding two-dimensional polyacrylamide gel electrophoresis technology and its uses in drug discovery and toxicology testing before the November 7, 1997 filing date of the Bandman '364 application. In particular the Celis article stated that "protein databases are expected to foster a variety of biological information.... -- among others, ... drug development and testing" (See Tab D, p. 2200, second column). The Franzen article shows that 2-D PAGE maps were used to identify proteins in clinical tumor material (See Tab E). The Bandman '364 application clearly discloses that VTP-1 is expressed "in various cDNA libraries, at least 42% of which are immortalized or cancerous, at least 24% of which involve immune response, and at least 29% are expressed in fetal/infant tissues or organs." (Bandman '364 application at page 14, lines 27-29.) The Bjellqvist article showed that a protein may be identified accurately by its positional co-ordinates, namely molecular mass and isoelectric point (See Tab F). The Bandman '364 application clearly disclosed SEQ ID NO:1 from which it would have been routine for one of skill in the art to predict both the molecular mass and the isoelectric point using algorithms well known in the art at the time of filing.

12. A person skilled in the art on November 7, 1997, who read the Bandman '364 application, would understand that application to disclose the SEQ ID NO:1 polypeptide to be highly useful in analysis of differential expression of proteins. For example, the specification of the Bandman '364 application would have led a person skilled in the art in November 7, 1997 who was using protein expression monitoring in connection with working on developing new drugs for the treatment of inflammation and disorders associated with cell proliferation and apoptosis to conclude that a 2-D PAGE map that used the isolated SEQ ID NO:1 polypeptide would be a highly useful tool and to request specifically that any 2-D PAGE map that was being used for such purposes utilize the SEQ ID NO:1 polypeptide sequence. Expressed proteins are useful for 2-D PAGE analysis in toxicology expression studies for a variety of reasons, particularly for purposes relating to providing controls for the 2-D PAGE analysis, and for identifying sequence or post-translational variants of the expressed sequences in response to exogenous compounds. Persons skilled in the art would appreciate that a 2-D PAGE map that utilized the SEQ ID NO:1 polypeptide sequence would be a more useful tool than a 2-D PAGE map that did not utilize this protein sequence in connection with conducting protein expression monitoring studies on proposed (or actual) drugs for treating inflammation and disorders associated with cell proliferation and apoptosis for such purposes as evaluating their efficacy and toxicity.

I discuss in more detail in items (a)-(c) below a number of reasons why a person skilled in the art, who read the Bandman '364 specification in November 7, 1997, would have concluded based on that specification and the state of the art at that time, that SEQ ID NO:1 polypeptide would be a highly useful tool for analysis of a 2-D PAGE map for evaluating the efficacy and toxicity of proposed drugs for inflammation and disorders associated with cell proliferation and apoptosis by means of 2-D PAGE maps, as well as for other evaluations:

(a) The Bandman '364 specification contains a number of teachings that would lead persons skilled in the art on November 7, 1997 to conclude that a 2-D PAGE map that utilized the substantially purified SEQ ID NO:1 polypeptide would be a more useful tool for gene expression monitoring applications relating to drugs for treating inflammation and disorders associated with cell proliferation and apoptosis than a 2-D PAGE map that did not use the SEQ ID NO:1 polypeptide sequence. Among other things, the Bandman '364 specification teaches that (i) the identity of the SEQ ID NO:1 polypeptide was determined from a THP-1 cell line cDNA library (THP1PEB01), (ii) the SEQ ID NO:1 polypeptide is the vesicle trafficking protein referred to as VTP-1, and (iii) VTP-1 is expressed in "various cDNA libraries, at least 42% of which are immortalized or cancerous, at least 24% of which involve immune response, and at least 29% are

expressed in fetal/infant tissues or organs" and therefore VTP-1 appears to play a role in inflammation and disorders associated with cell proliferation and apoptosis" and, therefore, VTP-1 appears to be involved in vesicle trafficking, and to play a role in inflammation and disorders associated with cell proliferation and apoptosis. (Bandman '364 application at p. 14, lines 5-29; see paragraph 9, *supra.*) The Bandman '364 application teaches that "VTP or a fragment or derivative thereof may be administered to a subject to prevent or treat a disorder associated with an increase in apoptosis. Such disorders include, but are not limited to . . . neurodegenerative diseases such as Alzheimer's disease. . . " (Bandman '364 application, page 28, lines 1-4.) The isolated polypeptide could therefore be used as a control to more accurately gauge the expression of VTP-1 in the sample and consequently more accurately gauge the affect of a toxicant on expression of the gene.

Moreover, the Bandman '364 specification teaches that SEQ ID NO:1 shares chemical and structural homology with mouse vacuolar protein-sorting homolog (mVps45) (GI 1703494). The VTP-1 and mVps45 share 97% sequence homology and have rather similar hydrophobicity plots (Bandman '364 application, page 14, lines 23-27, Figures 2A and 2B, and Figures 3A and 3B). mVps45 is a mammalian homolog to a yeast protein, Vps45, which "is essential for transport from the Golgi to a prevacuolar compartment." (Bandman '364 application, page 1, lines 16-17.) The Bandman '364 specification teaches that mammalian homologs to yeast vesicle trafficking proteins "are essential in mediating transport among the Golgi complex, synaptic vesicles, prelysosomal compartments, and the lysosome." (Bandman '364 specification, page 1, lines 24-26.)

(b) Also pertinent is that pre-November 7, 1997 article points to the potential role in Alzheimer's disease for proteins involved in lysosomal trafficking such as mammalian homologs (such as a human vps45) of yeast vps genes.

The Pevsner article (incorporated by reference into the Bandman '364 specification; Tab H) states that:

> A description of the proteins involved in lysosomal targeting is essential to understand lysosomal function in the biosynthetic and endocytic pathways, and also to understand diseases involving lysosomes. Protein trafficking to lysosomes may be disrupted in neurodegenerative disorders such as Alzheimer's disease and prion encephalopathies (Mayer et al., 1992; Cataldo et al., 1994) as well as organelle storage disorders diseases such as Chediak-Higashi syndrome (Zhao et al., 1994). (Tab H, page 14)

(c) Persons skilled in the art on November 7, 1997 would have appreciated (i)

that the protein expression monitoring results obtained using a 2-D PAGE map that utilized a SEQ ID NO:1 polypeptide would vary, depending on the particular drug being evaluated, and (ii) that such varying results would occur both with respect to the results obtained from the SEQ ID NO:1 polypeptide and from the 2-D PAGE map as a whole (including all its other individual proteins). These kinds of varying results, depending on the identity of the drug being tested, in no way detracts from my conclusion that persons skilled in the art on November 7, 1997, having read the Bandman '364 specification, would specifically request that any 2-D PAGE map that was being used for conducting protein expression monitoring studies on drugs for treating inflammation and disorders associated with cell proliferation and apoptosis (*e.g.*, a toxicology study or any efficacy study of the type that typically takes place in connection with the development of a drug) utilize the SEQ ID NO:1 polypeptide sequence. Persons skilled in the art on November 7, 1997 would have wanted their 2-D PAGE map to utilize the SEQ ID NO:1 polypeptide sequence because a 2-D PAGE map that utilized protein sequence information the polypeptide (as compared to one that did not) would provide more useful results in the kind of gene expression monitoring studies using 2-D PAGE maps that persons skilled in the art have been doing since well prior to November 7, 1997.

The foregoing is not intended to be an all-inclusive explanation of all my reasons for reaching the conclusions stated in this paragraph 12, and in paragraph 6, *supra*. In my view, however, it provides more than sufficient reasons to justify my conclusions stated in paragraph 6 of this Declaration regarding the Bandman '364 application disclosing to persons skilled in the art at the time of its filing substantial, specific and credible real-world utilities for the SEQ ID NO:1 polypeptide.

13. Also pertinent to my considerations underlying this Declaration is the fact that the Bandman '364 disclosure regarding the uses of the SEQ ID NO:1 polypeptide for protein expression monitoring applications is <u>not</u> limited to the use of that protein in 2-D PAGE maps. For one thing, the Bandman '364 disclosure regarding the technique used in gene and protein expression monitoring applications is broad. (Bandman '364 application at, e.g., page 25, lines 19-23 and page 38, lines 21-29.)

In addition, the Bandman '364 specification repeatedly teaches that the protein described therein (including the SEQ ID NO:1 polypeptide) may desirably be used in any of a number of long established "standard" techniques, such as ELISA or western blot analysis, for conducting protein expression monitoring studies. See, e.g.:

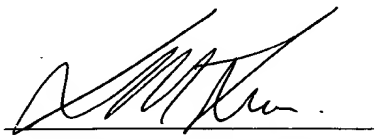
(a) Bandman '364 application at page 25, line 29 through page 26, line 2 ("A

variety of protocols for detecting and measuring the expression of VTP, using either polyclonal or monoclonal antibodies specific for the protein are known in the art. Examples include enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA), and fluorescence activated cell sorting (FACS)."); and

(b) Bandman '364 application at page 38, lines 21-29 ("A variety of protocols including ELISA, RIA, and FACS for measuring VTP are known in the art and provide a basis for diagnosing altered or abnormal levels of VTP expression. Normal or standard values for VTP expression are established by combining body fluids or cell extracts taken from normal mammalian subjects, preferably human, with antibody to VTP under conditions suitable for complex formation The amount of standard complex formation may be quantified by various methods, but preferably by photometric, means. Quantities of VTP expressed in subject, control and disease, samples from biopsied tissues are compared with the standard values. Deviation between standard and subject values establishes the parameters for diagnosing disease.").

Thus a person skilled in the art on November 7, 1997, who read the Bandman '364 specification, would have routinely and readily appreciated that the SEQ ID NO:1 polypeptide disclosed therein would be useful to conduct gene expression monitoring analyses using 2-D PAGE mapping or western blot analysis or any of the other traditional membrane-based protein expression monitoring techniques that were known and in common use many years prior to the filing of the Bandman '364 application. For example, a person skilled in the art in November 7, 1997 would have routinely and readily appreciated that the SEQ ID NO:1 polypeptide would be a useful tool in conducting protein expression analyses, using the 2-D PAGE mapping or western analysis techniques, in furtherance of (a) the development of drugs for the treatment of inflammation and disorders associated with cell proliferation and apoptosis, and (b) analyses of the efficacy and toxicity of such drugs.

14. I declare further that all statements made herein of my own knowledge are true and that all statements made herein on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, and that willful false statements may jeopardize the validity of this application and any patent issuing thereon.



L. Michael Furness, B.Sc.

Signed at Exning, United Kingdom
this 10th day of January 2002

N. Leigh Anderson
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Large Scale Biology Corporation, Rockville, MD

A two-dimensional gel database of rat liver proteins useful in gene regulation and drug effects studies

A standard two-dimensional (2-D) protein map of Fischer 344 rat liver (F344MST3) is presented, with a tabular listing of more than 1200 protein species. Sodium dodecyl sulfate (SDS) molecular mass and isoelectric point have been established, based on positions of numerous internal standards. This map has been used to connect and compare hundreds of 2-D gels of rat liver samples from a variety of studies, and forms the nucleus of an expanding database describing rat liver proteins and their regulation by various drugs and toxic agents. An example of such a study, involving regulation of cholesterol synthesis by cholesterol-lowering drugs and a high-cholesterol diet, is presented. Since the map has been obtained with a widely used and highly reproducible 2-D gel system (the Iso-Dalt® system), it can be directly related to an expanding body of work in other laboratories.

Contents

1 Introduction

High-resolution two-dimensional electrophoresis of proteins, introduced in 1975 by O'Farrell and others [1–4], has been used over the ensuing 16 years to examine a wide variety of biological systems, the results appearing in more than 5000 published papers. With the advent of computerized systems for analyzing two-dimensional (2-D) gel images and constructing spot databases, it is also possible to plan and assemble integrated bodies of information describing the appearance and regulation of thousands of protein gene products [5, 6]. Creating such databases involves amassing and organizing quantitative data from thousands of 2-D gels, and requires a substantial commitment in technology and resources.

Given the long-term effort required to develop a protein database, the choice of a biological system takes on considerable importance. While *in vitro* systems are ideal for answering many experimental questions, especially in cancer research and genetics, our experience with cell cultures and tissue samples suggests that some *in vivo* approaches could have major advantages. In particular, we have noticed that liver tissue samples from rats and mice appear to show greater quantitative reproducibility (in terms of individual protein expression) than replicate cell cultures. This is perhaps a natural result of the homeostasis maintained in a complete animal *vs.* the well-known variability of cell cultures, the latter due principally to differences in reagents (*e.g.*, fetal bovine serum), conditions (*e.g.*, pH) and genetic "evolution" of cell lines while in culture. It is also more difficult to generate adequate amounts of protein from cell culture systems (particularly with attached cells), forcing the investigator to resort to radioisotope-based or silver-based stain-detection methods. While these methods are more sensitive (sometimes much more sensitive) than the Coomassie Brilliant Blue (CBB) stain typically used for protein detection in "large" protein samples, they are generally more variable, more labor-intensive and, in the case of radiographic methods, may generate highly "noisy" images, due to the properties of the films used. By contrast, large protein samples can easily be prepared from liver using urea/Nonidet P-40 (NP-40) solubilization and stained with CBB, which has the advantage of being easily reproducible [8]. Finally, there remains the question of the "truthfulness" of many *in vitro* systems as compared to their *in vivo* analogs; how great are the changes caused by the introduction into a cul-

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Abbreviations: CBB, Coomassie Brilliant Blue; CPK, creatine phosphokinase; 2-D, two-dimensional; IEF, isoelectric focusing; MSN, master spot number; NP-40, Nonidet P-40; SDS, sodium dodecyl sulfate

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ture and the associated shift to strong selection for growth, and how do these affect experimental outcomes? Hence the apparent advantages of *in vitro* systems, in terms of experimental manipulation, may be counterbalanced by other factors relating to 2-D data quality.

There is a second important class of reasons for exploring the use of an *in vivo* biological system such as the liver. Historically, there have been two broad approaches to the mechanistic dissection of biochemical processes in intact cellular systems: genetics (a search for informative mutants) and the use of chemical agents (drugs and chemical toxins). Both approaches help us to understand complex systems by disrupting some specific functional element and showing us the result. With the development of techniques for genetic manipulation and cloning, the genetic approach can be effectively applied either *in vitro* or *in vivo*, although the *in vitro* route is usually quicker. The chemical approach can also be applied to either sort of biological system; here, however, the bulk of consistently acquired information is in experimental animals (rats and mice). While most biologists know a short list of compounds having specific, experimentally useful effects (*e.g.*, inhibitors of protein synthesis, ionophores, polymerase inhibitors, channel blockers, nucleotide analogs, and compounds affecting polymerization of cytoskeletal proteins), there is a much larger number of interesting chemically-induced effects, most of them characterized by toxicologists and pharmacologists in rodent systems. Just as a thorough genetic analysis would involve saturating a genome with mutations, it is possible to imagine a saturating number of drugs, the analysis of whose actions would reveal the complete biochemistry of the cell. While organized drug discovery efforts usually target specific desired effects, the nature of the process, with its dependence on screening large numbers of compounds, necessarily produces many unanticipated effects. It is therefore reasonable to suppose that the required broad range of compounds necessary to achieve "biochemical saturation" may be forthcoming; in fact, it may already exist among the hundreds of thousands of compounds that failed to qualify as drugs.

Among organs, the liver is an obvious choice for the study of chemical effects because of its well-known plasticity and responsiveness. The brain appears to be quite plastic (*e.g.* [7]), but it is a complicated mixture of cell types requiring skillful dissection for most experiments. The kidney, while quite responsive, also presents a potentially confounding mixture of cell types. The liver, by contrast, is made up of one predominant cell type which is easy to solubilize: the hepatocyte, representing more than 95% of its mass. Most importantly, the liver performs many homeostatic functions that require rapid modulation of gene expression. It appears that most chemical agents tested affect gene expression in the liver at some dosage (N. Leigh Anderson, unpublished observations), an interesting contrast to our earlier work with lymphocytes, for example, which seem to be much less responsive. Such results conform to the expectation that cells with a homeostatic, physiological role should be more plastic than cells differentiated for a purpose dependent on the action of a limited number of specific genes.

The liver also allows the parallels between *in vitro* and *in vivo* systems to be examined in detail. Significant progress has been made in the development of mouse, rat and human hepatocyte culture systems, as well as in precision-cut tissue slices. Using such an array of techniques, it is possible to assemble a matrix of mammalian systems including mouse and rat *in vivo* on one level and mouse, rat and human *in vitro* on a second level, and to compare effects between species and between systems. This approach allows us to draw informed conclusions regarding the biochemical "universality" of biological responses among the mammals and to offer some insight into the validity of *in vitro* approaches for toxicological screening. We believe this data will be necessary if *in vitro* alternatives are to achieve wide usage in government-mandated safety testing of drugs, consumer products and industrial and agricultural chemicals.

A number of interesting studies have been published using 2-D mapping to examine effects in the rodent liver. A number of investigarors have made use of the technique to screen for existing genetic variants [8–11] or induced mutations [12–14], mainly in the mouse. This work builds on the wealth of genetic information available on the mouse and its established position as a mammalian mutation-detection system. While some studies of chemical effects have been undertaken in the mouse [15–17], most have used the rat [18–23]. The examination of the cytochrome p-450 system, in particular, has been carried out almost exclusively on the rat [24, 25].

These considerations lead us to conclude that rodent liver offers the best opportunity to systematically examine an array of gene regulation systems, and ultimately to build a predictive model of large-scale mammalian gene control. The basic underlying foundation of such a project is a reliable, reproducible master 2-D pattern of liver, to which ongoing experimental results can be referred. In this paper, we report such a master pattern for the acidic and neutral proteins of rat liver (pattern F344MST3). In future, this master will be supplemented by maps of basic proteins, and analogous maps of mouse and human liver.

2 Materials and methods

2.1 Sample preparation

Liver is an ideal sample material for most biochemical studies, including 2-D analysis. A sample is taken of approximately 0.5 g of tissue from the apical end of the left lobe of the liver. Solubilization is effected as rapidly as practical; a delay of 5–15 min appears to cause no major alteration in liver protein composition if the liver pieces are kept cold (*e.g.*, on ice) in the interim. In the solubilization process, the liver sample is weighed, placed in a glass homogenizer (*e.g.*, 15 mL Wheaton); 8 volumes of solubilizing solution*

* The solubilizing solution is composed of 2% NP-40 (Sigma), 9 M urea (analytical grade, *e.g.*, BDH or Bio-Rad), 0.5% dithiothreitol (DTT; Sigma) and 2% carrier ampholytes (pH 9–11 LKB; these come as a 20% stock solution, so 2% final concentration is achieved by making the final solution 10% 9–11 Ampholine by volume). A large batch of solubilizer (several hundred mL) is made and stored frozen at −80°C in aliquots sufficient to provide enough for one day's estimated sample preparation requirement. The solution is never allowed to become warmer than room temperature at any stage during preparation or thawing for use, since heating of concentrated urea solutions can produce contaminants that covalently modify proteins producing artifactual charge shifts. Once thawed, any unused solubilizer is discarded.

ded (*i.e.*, 4 mL per 0.5 g tissue) and the mixture is homogenized using first the loose- and then then the tight-fitting glass pestle. This takes approximately 5 strokes with each pestle and is carried out at room temperature because it would crystallize out in the cold. Once the liver sample is thoroughly homogenized in the solubilizer, it is assumed that all the proteins are denatured (by the chaotropic effect of the urea and NP-40 detergent) and the enzymes inactivated by the high pH (~9.5). Therefore these samples may be kept at room temperature until they can be centrifuged or frozen as a group (within several hours of preparation). The samples are centrifuged for 6×10^7 *g* min (*e.g.*, 500 000 *g* for 12 min using a Beckman TL-100 centrifuge). The centrifuge rotor is maintained at just below room temperature (*e.g.*, 15–20°C), but not too cold, so as to prevent the precipitation of urea. The centrifuge of choice is a Beckman TL-100 because of the sample tube sizes available, but any ultracentrifuge accepting smallish tubes will suffice. When an appropriate centrifuge is not available near the site of sample preparation, samples can be frozen at −80°C and thawed prior to centrifugation and collection of supernatants. Each supernatant is carefully removed following centrifugation and aliquoted into at least 4 clean tubes for storage. This is done by transferring all the supernatant to one clean tube, mixing this gently (to assure homogeneous composition) and then dividing it into 4 aliquots. The aliquots are frozen immediately at −80°C. These multiple aliquots can provide insurance against a failed run or a freezer breakdown.

2.2 Two-dimensional electrophoresis

Sample proteins are resolved by 2-D electrophoresis using the 20 × 25 cm Iso-Dalt® 2-D gel system ([26–29]; produced by LSB and by Hoefer Scientific Instruments, San Francisco) operating with 20 gels per batch. All first-dimensional isoelectric focusing (IEF) gels are prepared using the same single standardized batch of carrier ampholytes BDH 4–8A in the present case, selected by LSB's batch-testing program for rat and mouse database work**). A 10 µL sample of solubilized liver protein is applied to each gel, and the gels are run for 33 000 to 34 500 volt-hours using a progressively increasing voltage protocol implemented by a programmable high-voltage power supply. An Angelique™ computer-controlled gradient-casting system (produced by LSB) is used to prepare second-dimensional sodium dodecyl sulfate (SDS) polyacrylamide gradient slab gels in which the top 5% of the gel is 11%T acrylamide, and the lower 95% of the gel varies linearly from 11% to 18%T.

This system has recently been modified so as to employ a commercially available 30.8%T acrylamide/*N,N'*-methylenebisacrylamide prepared solution (thus avoiding the handling of the solid acrylamide monomer) and three additional stock solutions: buffer (made from Sigma pre-set Tris), persulfate and *N,N,N',N'*-tetramethylethylenediamine (TEMED). Each gel is identified by a computer-printed filter paper label polymerized into the lower left corner of the gel. First-dimensional IEF tube gels are loaded directly (as extruded) onto the slab gels without equilibration, and held in place by polyester fabric wedges (Wedgies™, produced by LSB) to avoid the use of hot agarose. Second-dimensional slab gels are run overnight, in groups of 20, in cooled DALT tanks (10°C) with buffer circulation. All run parameters, reagent source and lot information, and notations of deviation from expected results are entered by the technician responsible on a detailed, multi-page record of the experiment.

2.3 Staining

Following SDS-electrophoresis, slab gels are stained for protein using a colloidal Coomassie Blue G-250 procedure in covered plastic boxes, with 10 gels (totalling approximately 1 L of gel) per box. This procedure (based on the work of Neuhoff [30, 31]) involves fixation in 1.5 L of 50% ethanol and 2% phosphoric acid for 2 h, three 30 min washes, each in 2 L of cold tap water, and transfer to 1.5 L of 34% methanol, 17% ammonium sulfate and 2% phosphoric acid for 1 h, followed by the addition of a gram of powdered Coomassie Blue G-250 stain. Staining requires approximately 4 days to reach equilibrium intensity, whereupon gels are transferred to cool tap water and their surfaces rinsed to remove any particulate stain prior to scanning. Gels may be kept for several months in water with added sodium azide. The water washes remove ethanol that would dissolve the stain (and render the system noncolloidal, with high backgrounds). The concentrated ammonium sulfate and methanol solution is diluted by equilibration with the water volume of the gels to automatically achieve the correct final concentrations for colloidal staining. Practical advantages of this staining approach can be summarized as follows: (i) the low, flat background makes computer evaluation of small spots (max OD < 0.02) possible, especially when using laser densitometry; (ii) up to 1500 spots can be reliably detected on many gels (*e.g.*, rat liver) at loadings low enough to preserve excellent resolution; and (iii) reproducibility appears to be very good: at least several hundred spots have coefficients of reproducibility less than 15%. This value is at least as good as previous CBB methods, and significantly better than many silver stain systems.

2.4 Positional standardization

The carbamylated rabbit muscle creatine phosphokinase (CPK) standards [32] are purchased from Pharmacia and BDH. Amino acid compositions, and numbers of residues present in proteins used for internal standardization, are taken from the Protein Identification Resource (PIR) sequence database [33].

2.5 Computer analysis

Stained slab gels are digitized in red light at 134 micron resolution, using either a Molecular Dynamics laser scanner (with pixel sampling) or an Eikonix 78/99 CCD scanner. Raw digitized gel images are archived on high-density DAT tape (or equivalent storage media) and a greyscale videoprint prepared from the raw digital image as hard-copy backup of the gel image. Gels are processed using the Kepler® software system (produced by LSB), a commercially available workstation-based software package built on

** This material (succeeding certified batches of which are available from Hoefer Scientific Instruments) has the most linear pH gradient produced by any ampholyte tested except for the Pharmacia wide range which has an unacceptable tendency to bind high-molecular weight acidic proteins, causing them to streak).

some of the principles of the earlier TYCHO system [34–41]. Procedure PROC008 is used to yield a spotlist giving position, shape and density information for each detected spot. This procedure makes use of digital filtering, mathematical morphology techniques and digital masking to remove the background, and uses full 2-D least-squares optimization to refine the parameters of a 2-D Gaussian shape for each spot. Processing parameters and file locations are stored in a relational database, while various log files detailing operation of the automatic analysis software are archived with the reduced data. The computed resolution and level of Gaussian convergence of each gel are inspected and archived for quality control purposes.

Experiment packages are constructed using the Kepler experiment definition database to assemble groups of 2-D patterns corresponding to the experimental groups (*e.g.*, treated and control animals). Each 2-D pattern is matched to the appropriate "master" 2-D pattern (pattern F344MST3 in the case of Fischer 344 rat liver), thereby providing linkage to the existing rodent protein 2-D databases. The software allows experiments containing hundreds of gels to be constructed and analyzed as a unit, with up to 100 gels displayed on the screen at one time for comparative purposes and multiple pages to accommodate experiments of > 1000 gels. For each treatment, proteins showing significant quantitative differences vs. appropriate controls are selected using group-wise statistical parameters (*e.g.*, Student's t-test, Kepler[3] procedure STUDENT). Proteins satisfying various quantitative criteria (such as $P < 0.001$ difference from appropriate controls) are represented as highlighted spots onscreen or on computer-plotted protein maps and stored as spot populations (*i.e.*, logical vectors) in a liver protein database. Quantitative data (spot parameters, statistical or other computed values) are stored as real-valued vectors in the database. Analysis of coregulation is performed using a Pierson product-moment correlation (Kepler procedure CORREL) to determine whether groups of proteins are coordinately regulated by any of the treatments. Such groups can be presented graphically on a protein map, and reported together with the statistical criteria used to assess the level of coregulation. Multivariate statistical analysis (*e.g.*, principal components' analysis) is performed on data exported to SAS (SAS Institute).

2.6 Graphical data output

Graphical results are prepared in GKS and translated within Kepler[6] into output for any of a variety of devices. Linedrawing output is typically prepared as Postscript and printed on an Apple Laserwriter. Detailed maps presented here have been generated using an ultra-high-resolution Postscript-compatible Linotronic output device. Greyscale graphics are reproduced from the workstation screen using a Seikosha videoprinter. Patterns are shown in the standard orientation, with high molecular mass at the top and acidic proteins to the left.

2.7 Experiment LSBC04

In the study described here 12-week-old Charles River male F344 rats were used. Diets were prepared at LSB, based on a Purina 5755M Basal Purified Diet. Lovastatin and cholestyramine were obtained as prescription pharmaceuticals, ground and mixed with the diet at concentrations of 0.075% and 1%, respectively. The high cholesterol diet was Purina 5801M-A (5% cholesterol plus 1% sodium cholate in the control diet). Animal work was carried out by Microbiological Associates (Bethesda, MD). Animals were acclimatized for one week on the control diet, fed test or control diets for one week, and sacrificed on day 8. Average daily doses of lovastatin and cholestyramine in appropriate groups were 37 mg/kg/day and 5 g/kg/day, respectively, based on the weight of the food consumed. Liver samples were collected and prepared for 2-D electrophoresis according to the standard liver protocol (homogenization in 8 volumes of 9 M urea, 2% NP-40, 0.5% dithiothreitol, 2% LKB pH 9–11 carrier ampholytes, followed by centrifugation for 30 min at 80 000 × *g*). Kidney, brain and plasma samples were frozen. Gels were run as described above, and the data was analyzed using the Kepler[3] system. Gels were scaled, to remove the effect of differences in protein loading, by setting the summed abundances of a large number of matched spots equal for each gel (linear scaling).

3 Results and discussion

3.1 The rat liver protein 2-D map

F344MST3 is a standard 2-D pattern of rat liver proteins, based on the Fischer 344 strain. This pattern was initiated from a single 2-D gel and extensively edited in an experiment comparing it to a range of protein loads, so as to include both small spots and well-resolved representations of high-abundance spots. More than 700 rat liver 2-D patterns have been matched to F344MST3 in a series of drug effects and protein characterization experiments, and numerous new spots (induced by specific drugs, for instance) have been added as a result. A modified version including additional spots present in the Sprague-Dawley outbred rat has also been developed (data not shown). Figure 1 shows a greyscale representation and Fig. 2 a schematic plot of the master pattern. More than 1200 spots are included, most of which are visible on typical gels loaded with 10 µL of solubilized liver protein prepared by the standard method and stained with colloidal Coomassie Blue. Master spot numbers (MSN's) have been assigned to all proteins, and appear in the following figures, each showing one quadrant of the pattern. Figure 3 shows the upper left (acidic, high molecular mass) quadrant, Fig. 4 the upper right (basic, high molecular mass) quadrant, Fig. 5 the lower left (acidic, low molecular mass) quadrant, and Fig. 6 the lower right (basic, low molecular mass) quadrant. The quadrants overlap as an aid to moving between them. The gel position (in 100 micron units), isoelectric point (relative to the CPK internal p*I* standards) and SDS molecular mass (from the calibration curve in Fig. 8) are listed for each spot (Table 1). Because of the precision of the CPK-p*I* values, these parameters can be used to relate spot locations between gel systems more reliably than using p*I* measurements expressed as pH. A major objective of current studies is the identification of all major spots corresponding to known liver proteins, as well as rigorous definitions of subcellular organelle contents. Of particular interest to us is the parallel development of identifications in the rat and mouse liver maps, allowing detailed comparisons of gene expression effects in the two systems. The results of these studies will be presented systematically in a later edition of this database.

we include here a useful series of 22 orienting identifications as an aid to other users of the rat liver pattern (Table

2 Carbamylated charge standards, computed p*I*'s and molecular mass standardization

We have previously shown that the use of a system of close-spaced internal p*I* markers (made by carbamylating a basic protein) offers an accurate and workable solution to the problem of assigning positions in the p*I* dimension [32]. The same system, based on 36 protein species made by carbamylating rabbit muscle CPK, has been used here to assign p*I*'s to most rat liver acidic and neutral proteins. The standards were coelectrophoresed with total liver proteins, and the standard spots added to a special version of the master pattern F344MST3. The gel *X*-coordinates of all liver protein spots lying within the CPK charge train were then transformed into CPK p*I* positions by interpolation between the positions of immediately adjacent standards (Table 1) using a Kepler[2] vector procedure.

It has proven possible to compute fairly accurate p*I* values for many proteins from the amino acid composition [42]. We have attempted here to test a further elaboration of this approach, in which we computed p*I*'s for the CPK standards themselves, based on our knowledge of the rabbit muscle CPK sequence and the fact that adjacent members of the charge train typically differ by blockage of one additional lysine residue (Table 3). We compared these values to similar computed p*I*'s for an additional set of carbamylated standards made from human hemoglobin beta chains and a series of rat liver and human plasma proteins of known position and sequence (Fig. 7, Table 4). The result demonstrates good concordance between these systems. Two proteins show significant deviations: liver fatty-acid binding protein (FABP; #1 in Table 4) and protein disulphide isomerase (#20 in the table). The FABP spot present on F344MST3 may represent a charge-modified version of a more basic parent spot closer to the expected p*I*, not resolved in the IEF/SDS gel. Of particular importance is the fact that, by comparing computed p*I*'s of sequenced but unlocated proteins with the CPK p*I*'s, we can assign a probable gel location without making any assumptions regarding the actual gel pH gradient. This offers a useful shortcut, given the vagaries of pH measurement on small diameter IEF gels. We have used this approach to compute the CPK p*I*'s of all rat and mouse proteins in the PIR sequence database, as an aid to protein identification (data not shown).

In order to standardize SDS molecular weight (SDS-MW), we have used a standard curve fitted to a series of identified proteins (Fig. 8). Rather than using molecular mass *per se*, we have elected to use the number of amino acids in the polypeptide chain, as perhaps a better indication of the length of the SDS-coated rod that is sieved by the second dimension slab. The resulting values were multiplied by 112 (the weighted average mass of amino acids in sequenced proteins) to give predicted molecular masses. Because we use gradient slabs, we have not constrained the fitted curve to conform to any predetermined model; rather we tried many equations and selected the best using the program "Tablecurve" on a PC. The equation chosen was $y = a + bx + c/x^2$, where y is the number of residues, x is the gel *Y* coordinate, a is 511.83, b is −0.2731 and c is 33183801. The resulting fit appears to be fairly good over a broad range of molecular mass.

3.3 An example of rat liver gene regulation: Cholesterol metabolism

Experiment LSBC04 was designed as a small-scale test of the regulation of cholesterol metabolism *in vivo* by three agents included in the diet: lovastatin (Mevacor®, an inhibitor of HMG-CoA reductase); cholestyramine (a bile acid sequestrant that has the effect of removing cholesterol from the gut-liver recirculation); and cholesterol itself. The first two agents should lower available cholesterol and the third should raise it, allowing manipulation of relevant gene expression control systems in both directions. Such an experiment offers an interesting test of the 2-D mapping system since most of the pathway enzymes are present in low abundance, many are membrane-bound and difficult to solubilize, and the pathway itself is complex. Approximately 1000 proteins were separated and detected in liver homogenates. Twenty-one proteins were found to be affected by at least one treatment, and these could be divided into several coregulated groups.

3.3.1 MSN 413 (putative cytosolic HMG-CoA synthase) and sets of spots regulated coordinately or inversely

One group of spots (including a spot assigned to the cytosolic HMG-CoA synthase, MSN 413) showed the expected increase in abundance with lovastatin or cholestyramine, the synergistic further increase with lovastatin and cholestyramine, and a dramatic decrease with the high cholesterol diet. Spot number 413 is the most strongly regulated protein in the present experiment, showing a 5- to 10-fold induction after a 1 week treatment with 0.075% lovastatin and 1% cholestyramine in the diet (Figs. 9 and 10). Its expression follows precisely the expectation for an enzyme whose abundance is controlled by the cholesterol level; it is progressively increased from the control levels by cholestyramine, lovastatin and lovastatin plus cholestyramine, and it sinks below the threshold of detection in animals fed the high cholesterol diet. This spot has been tentatively identified as the cytosolic HMG-CoA synthase, based on a reaction with an antiserum to that protein provided by Dr. Michael Greenspan at Merck Sharp & Dohme Research Laboratories. This enzyme lies immediately before HMG-CoA reductase in the liver cholesterol biosynthesis pathway, and is known to be co-regulated with it. Spot 413 has an SDS molecular weight of about 54 000 and a CPK p*I* of −11.4, in reasonably close agreement with a molecular weight of 57 300 and a CPK p*I* of −15.7 computed from the known sequence of the hamster enzyme [43].

Using a classical product-moment correlation test (Kepler procedure CORREL), a series of five additional spots was found to be coregulated with 413. The level of correlation was exceedingly high (> 95%). Two of these, 1250 and 933, are at similar molecular weights and approximately one charge more acidic than 413 (Fig. 9), indicating that they may be covalently modified forms of the 413 polypeptide. This suspicion is strengthened by the observation that both spots are also stained by the antibody to cytosolic HMG-CoA synthase. The remaining three correlated spots appear

to comprise an additional related pair (1253 and 1001) of around 40 kDa and a single spot (1119) of around 28 kDa. Because these two presumed proteins are present at substantially lower abundances than 413, and because the cytosolic HMG-CoA synthase is reported to consist of only one type of polypeptide, they are likely to represent other, very tightly coregulated enzymes. A second group of six spots was selected based on a regulatory pattern close to the inverse of that for spot 413 (MSN's 34, 79, 178, 182, 204, 347; data not shown). For these proteins, the lowest level of expression occurs with exposure to lovastatin plus cholestyramine and the highest level upon exposure to the high-cholesterol diet. Spots 182 and 79 are highly correlated and lie about one charge apart at the same molecular weight; they may thus be isoforms of a single protein. The other four spots probably represent additional enzymes or subunits.

3.3.2 MSN 235 and coregulated spots

A third group of five spots, mainly comprised of mitochondrial proteins including putative mitochondrial HMG-CoA synthase spots, showed a modest induction by lovastatin alone, but little or no effect with any of the other treatments (including the combination of lovastatin and cholestyramine; Fig. 12). This result is intriguing because lovastatin was expected to affect only the regulation of enzymes of cholesterol synthesis, which is entirely extra-mitochondrial. Three of the spots (235, 134, 144) form a closely-packed triad at approximately 30 kDa, and are likely to represent isoforms of one protein. All three spots are stained by an antibody to the mitochondrial form of HMG-CoA synthase obtained from Dr. Greenspan. Subcellular fractionation indicates a mitochondrial location. The other two spots (633 at about 38 kDa and 724 at about 69 kDa) are each present at lower abundance than the members of the triad.

3.3.3 An example of an anti-synergistic effect

A sixth spot (367) shows strong induction by lovastatin (two- to threefold), and about half as much induction with lovastatin plus cholestyramine, but without sharing the animal-animal heterogeneity pattern of the 235-set (Fig. 13). This protein is also mitochondrial, and represents the clearest example of an anti-synergistic effect of lovastatin and cholestyramine. The existence of such an effect demonstrates that lovastatin and cholestyramine do not act exclusively through the same regulatory pathway.

3.3.4 Complexity of the cholesterol synthesis pathway

Taken together, these results suggest that treatment with lovastatin alone can affect both cytosolic and mitochondrial pathways using HMG-CoA, while cholestyramine, on the other hand, either alone or in combination with lovastatin, produces a strong effect on the putative cytosolic pathway, but little or no effect on the putative mitochondrial pathway. An explanation for this difference may lie in lovastatin's effect on levels of HMG-CoA and related precursor compounds that are exchanged between the cytosol and the mitochondrion, whereas cholestyramine should affect only the cytosolic pathways directly controlled by cholesterol and bile acid levels. It remains to be explained why some proteins of the putative mitochondrial pathway are so much more variable in their expression in all groups. An examination of all the coregulated groups suggests that quantitative statistical techniques can extract a wealth of interesting information from large sets of reproducible gels. The abundance of spots in the 413 coregulation group, for example, shows an amazing level of concordance in their relative expression among the five individuals of the lovastatin and cholestyramine treatment group. This effect is not due to differences in total protein loading, since they have already been removed by scaling, and since proteins with quite different regulation patterns can be demonstrated (*e.g.*, Fig. 13). Such effects raise the possibility that many gene coregulation sets may be revealed through the study of a sufficiently large population of control animals (*i.e.*, without any experimental manipulation). This approach, exploiting natural biological variation in protein expression instead of drug effects, offers an important incentive for the construction of a large library of control animal patterns.

4 Conclusions

Because of the widespread use of rat liver in both basic biochemistry and in toxicology, there is a long-term need for a comprehensive database of liver proteins. The rat liver master pattern presented here has proven to be an accurate representation of this system, having been matched to more than 700 gels to date. As the number of proteins identified and the number of compounds tested for gene expression effects grows, we expect this database to contribute valuable insights into gene regulation. Its practical utility in several areas of mechanistic toxicology is already being demonstrated.

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6 Addendum 1: Figures 1–13

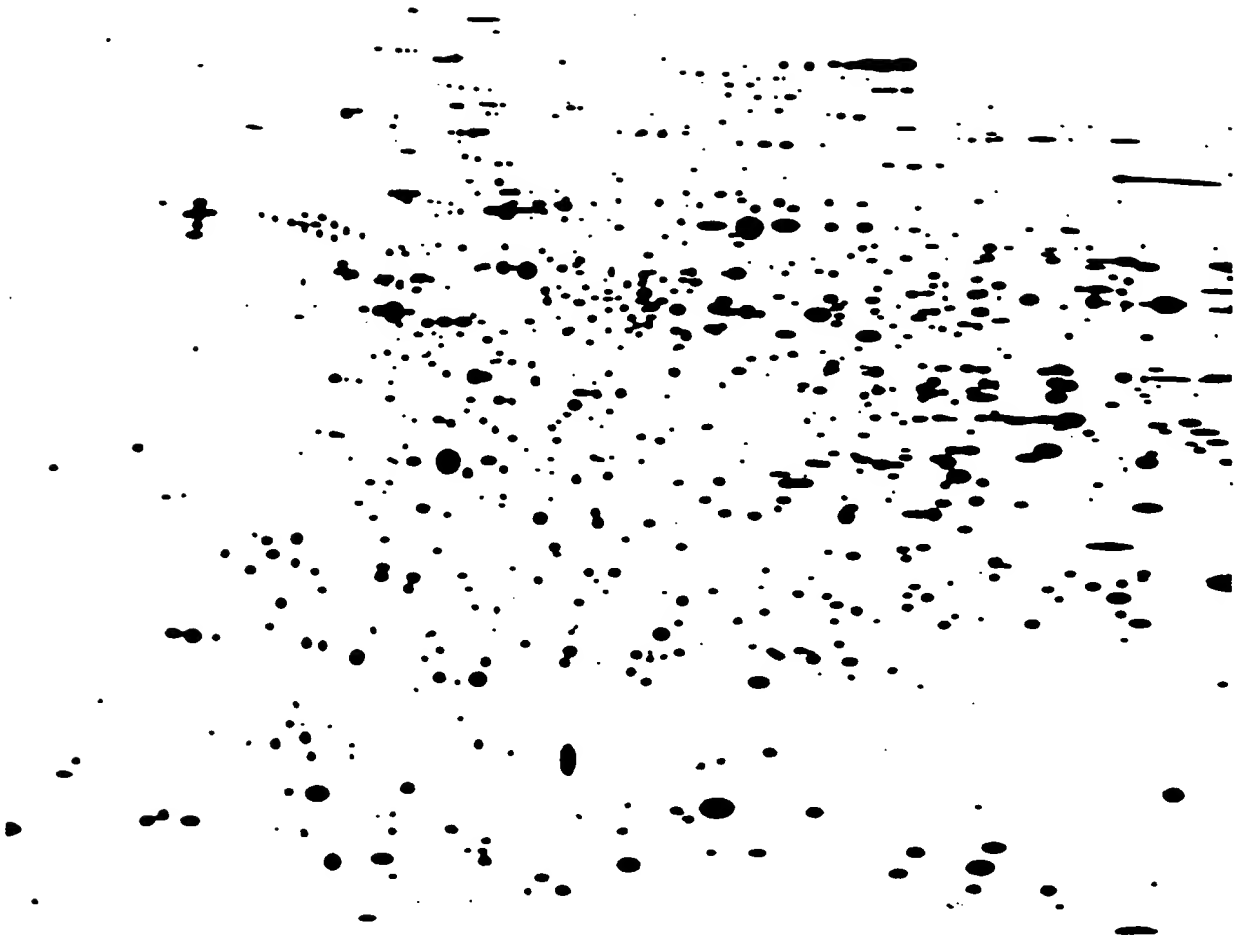
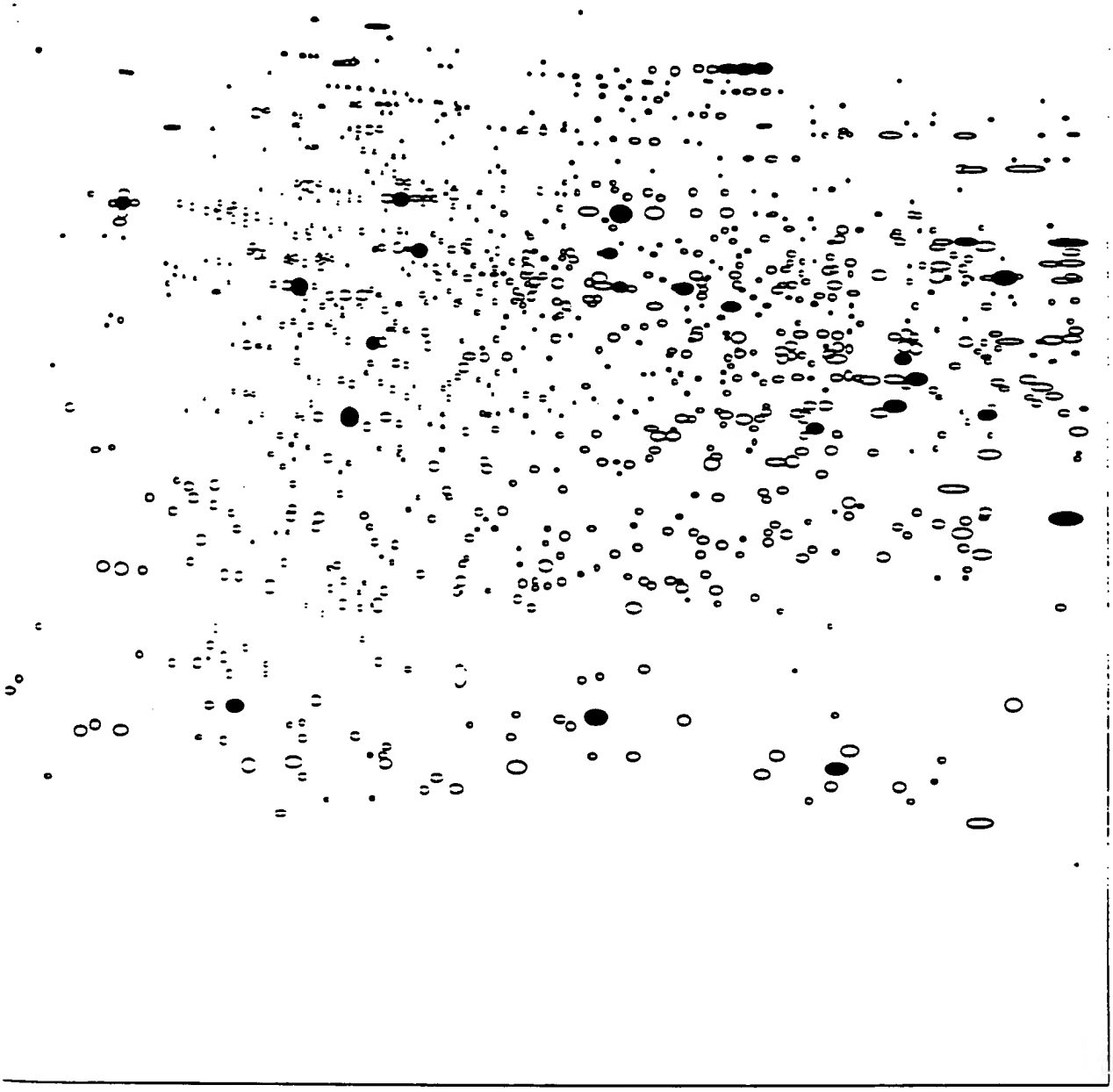


Figure 1. Synthetic representation of the standard rat liver 2-D master pattern, rendered as a greyscale image using a videoprinter.



re 2. Schematic representation of the master pattern (the same as Fig. 1), useful as an aid in relating specific areas of Fig. 1 and the following detailed
lrants.

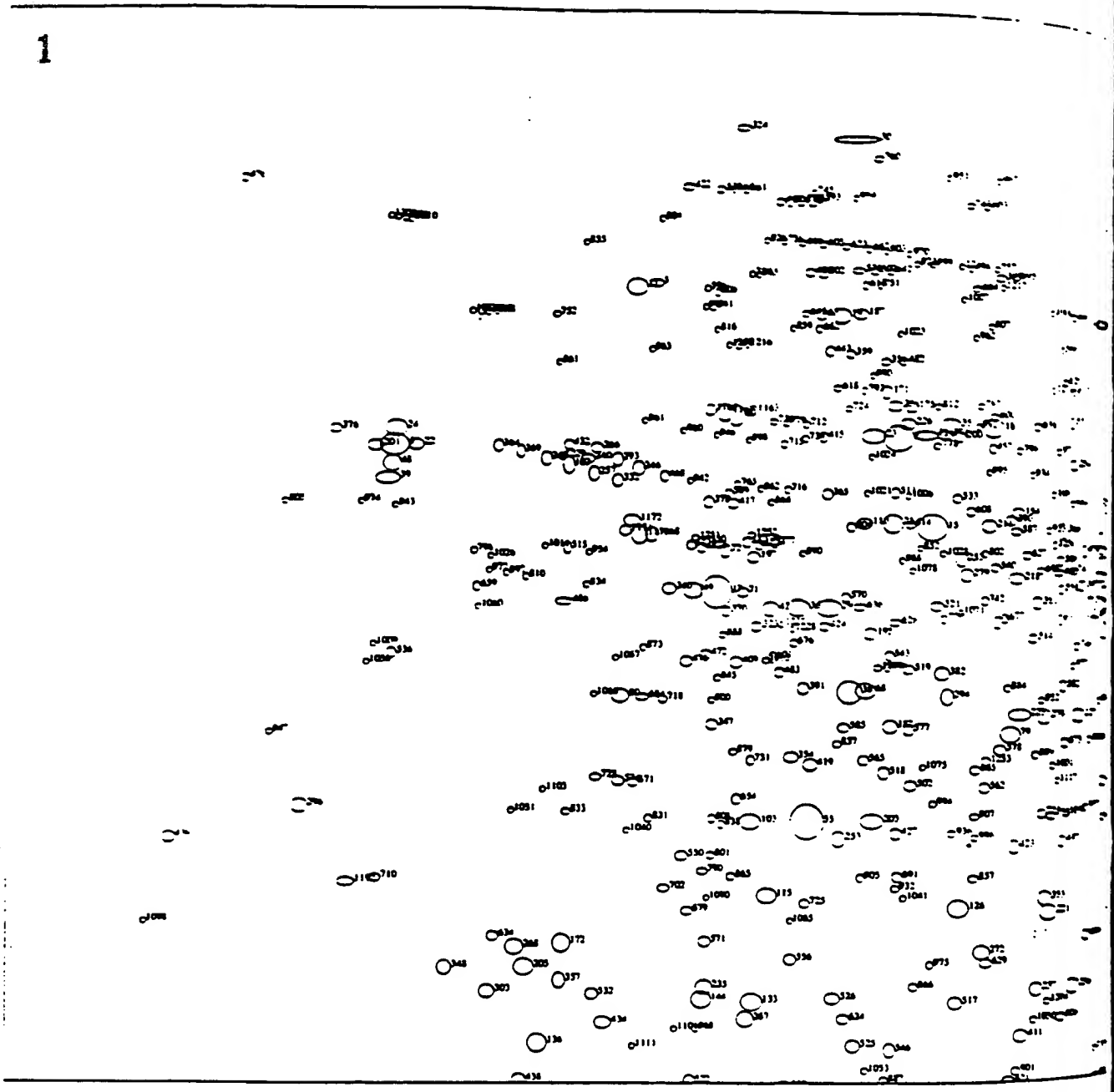


Figure 3. Upper left (high molecular weight, acidic) quadrant (#1) of the rat liver map, showing spot numbers.

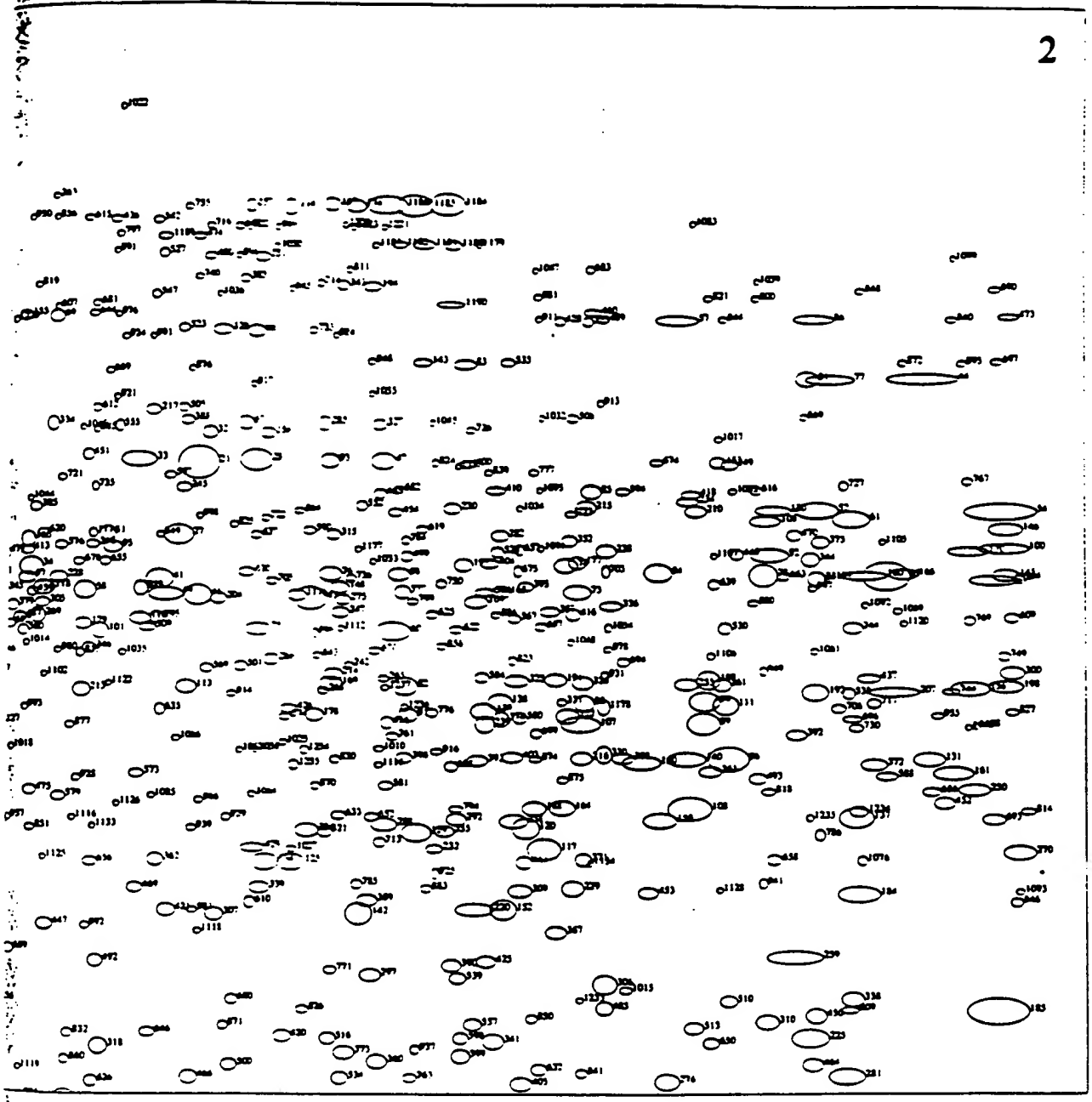


Figure 4. Upper right (high molecular weight, basic) quadrant (#2) of the rat liver map, showing spot numbers.

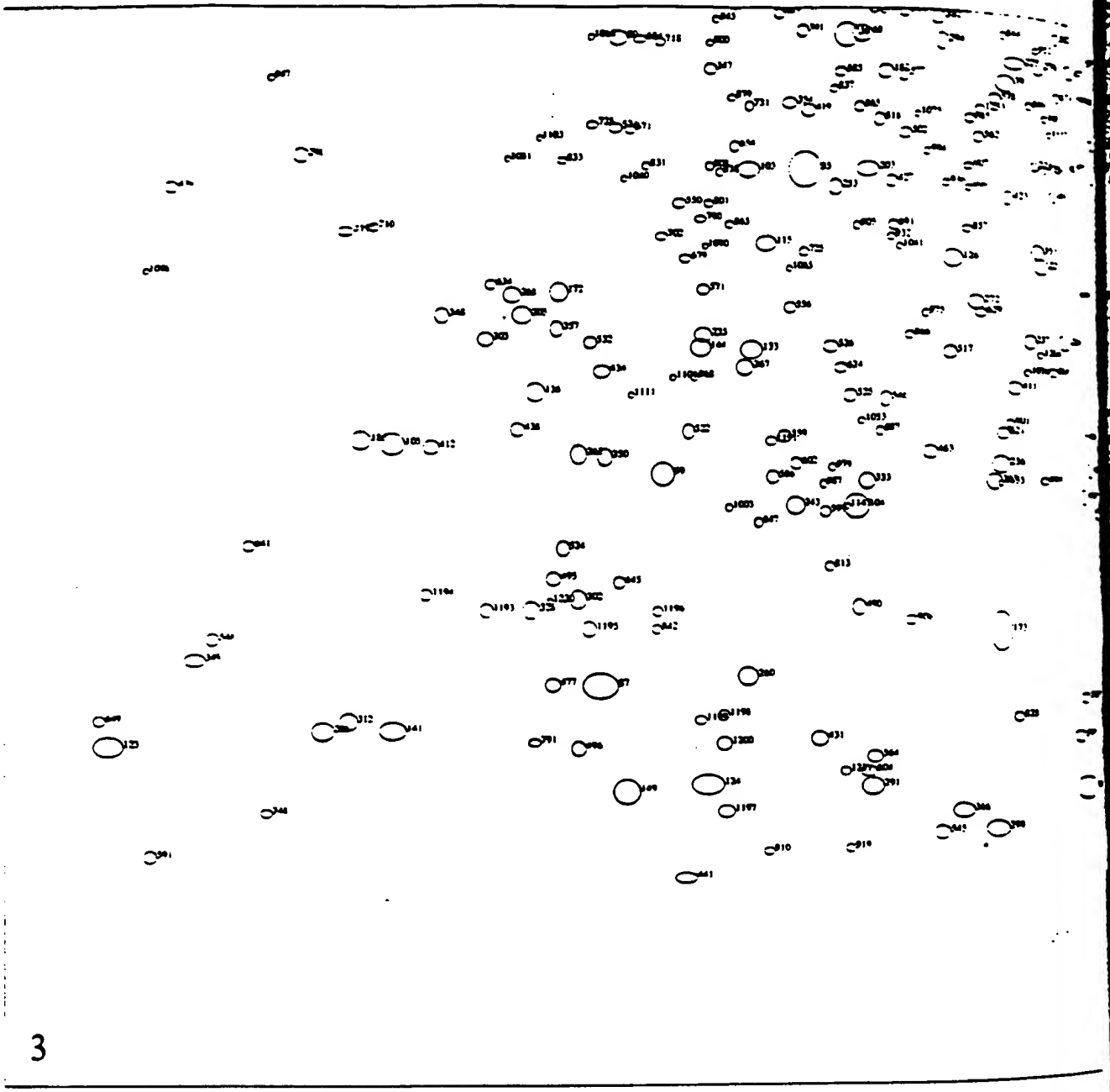
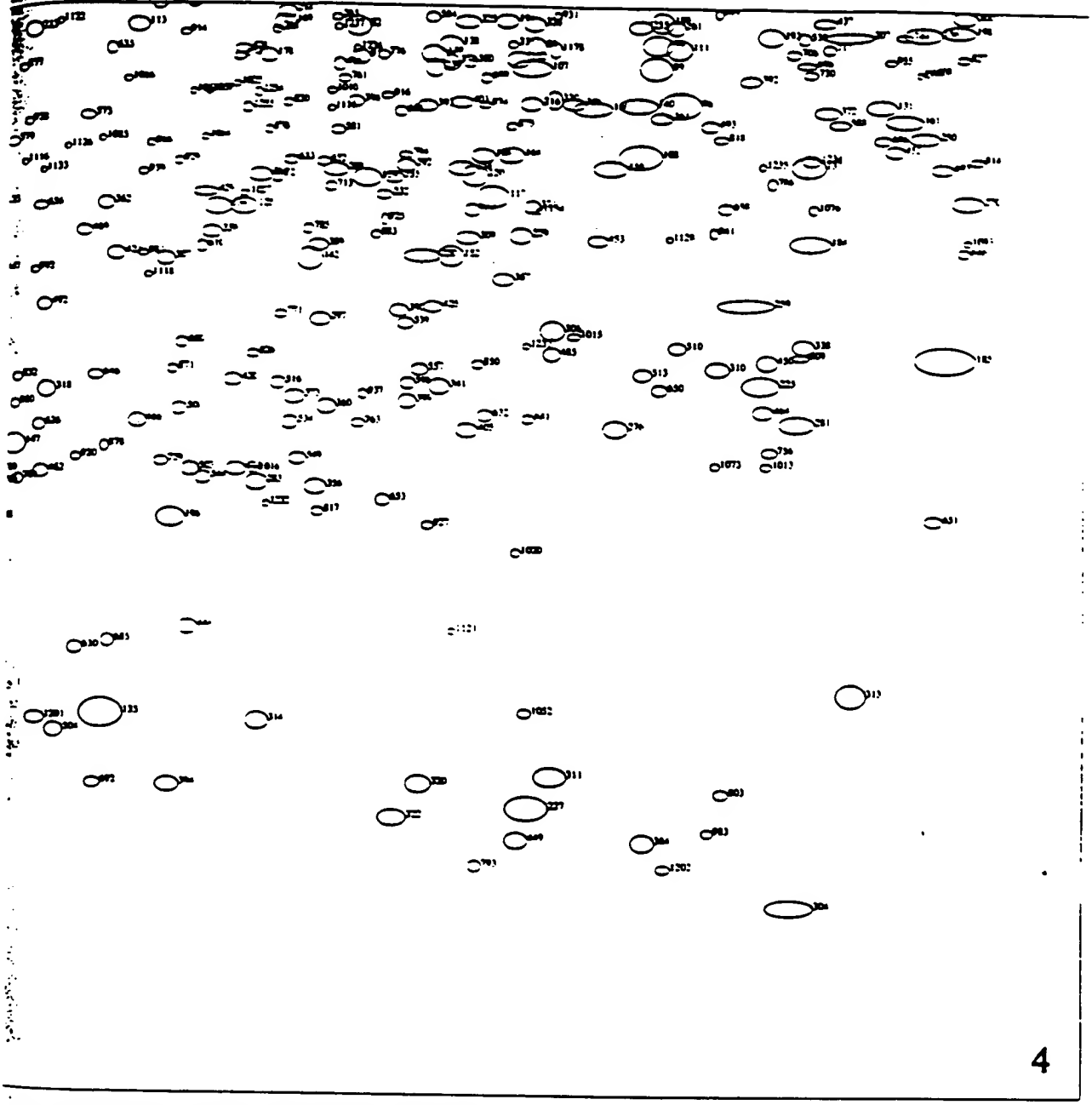
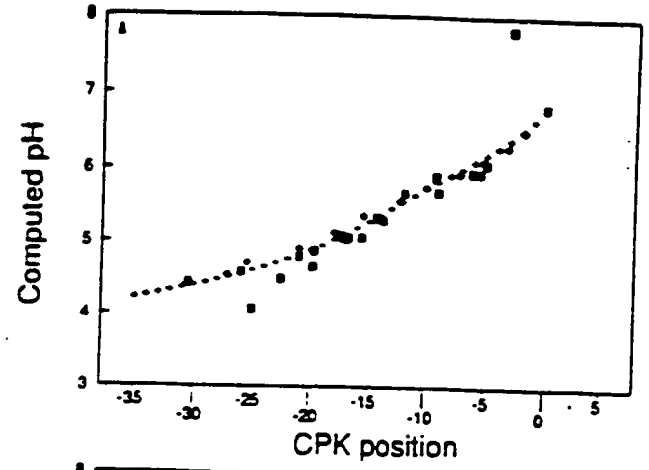


Figure 5. Lower left (low molecular weight, acidic) quadrant (#3) of the rat liver map, showing spot numbers.



ure 6. Lower right (low molecular weight, basic) quadrant (#4) of the rat liver map, showing spot numbers.



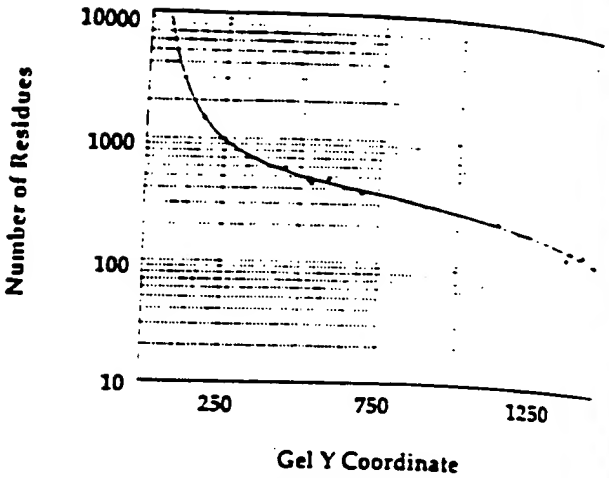
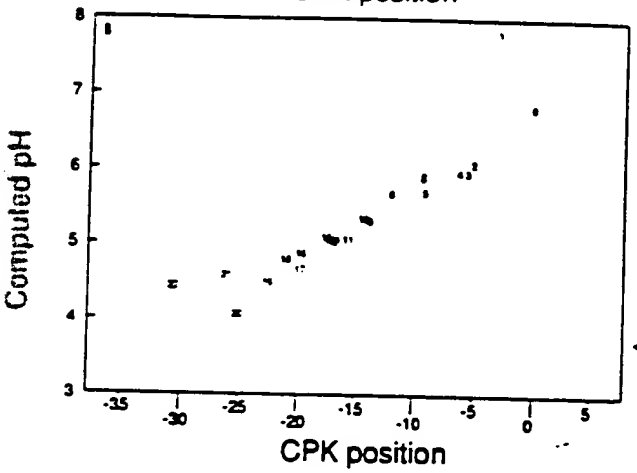


Figure 8. Plot of number of amino acids versus gel Y-position, with fitted curve used to predict molecular mass of unidentified proteins.



◀ *Figure 7.* (a) Plot of computed isoelectric point versus gel X-position for two sets of carbamylated standard proteins (rabbit muscle CPK [+] and human hemoglobin β chain, filled diamonds) and several other proteins (shaded squares). (b) The identities of the various proteins represented by the squares are indicated by the numbers in corresponding positions on (a); these refer to Table 4.

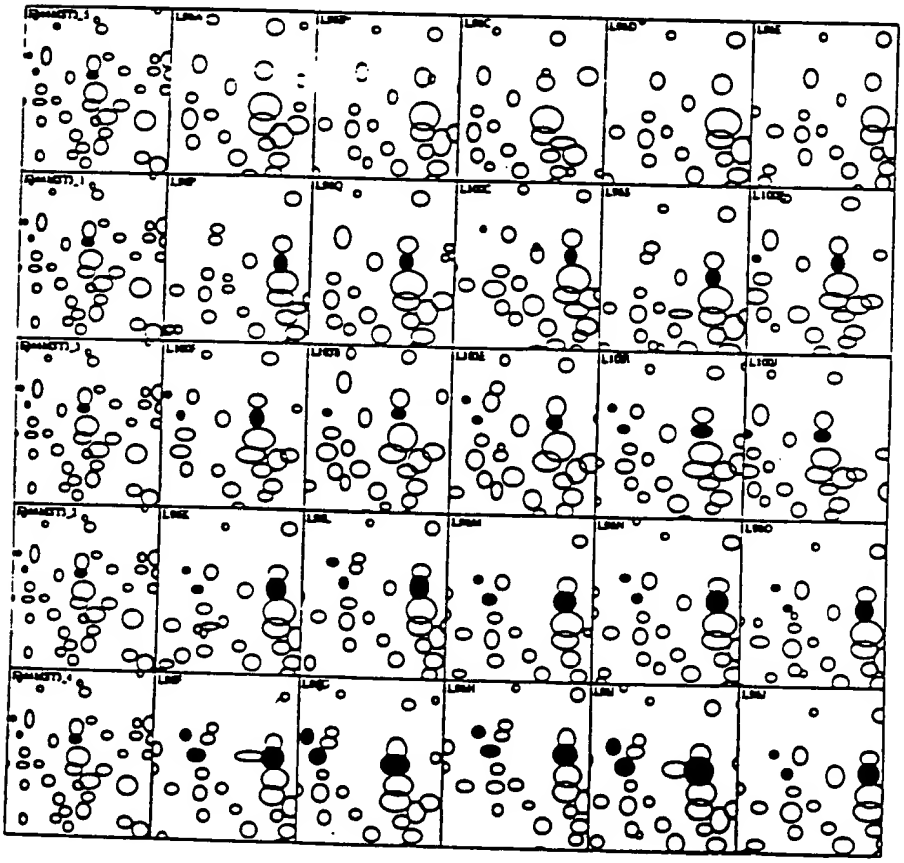


Figure 9. Montage showing effects in the region of MSN:413. The montage shows a small window into one portion of the 2-D pattern, one row of windows for each experimental group, and one panel for each gel in the experiment. The left-most pattern in each row is a group-specific copy of the master pattern followed by the patterns for the five individual rats in the group. The highlighted protein spots (filled circles) are spot 413 (on the right of each panel; identified as cytosolic HMG-CoA synthase) and two modified forms of it (1250 and 933). From the top, the rows (experimental groups) are: high cholesterol, controls, cholestyramine, lovastatin, and lovastatin plus cholestyramine.

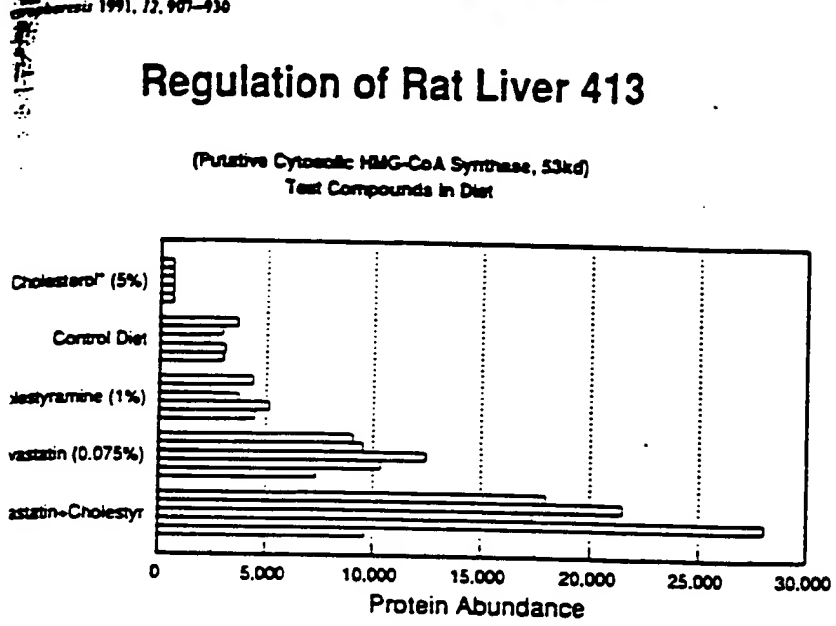


Figure 10. Bargraph showing the quantitative effects of various treatments on the abundance of MSN:413 (cytosolic HMG-CoA synthase) in the gels of Fig. 9.

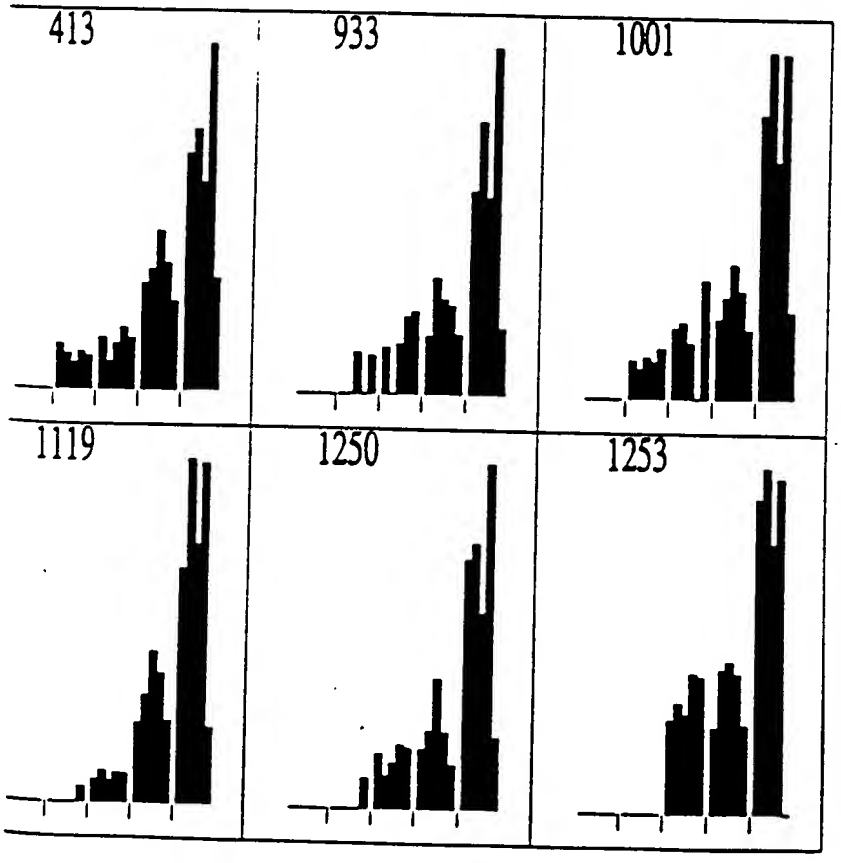


Figure 11. Bargraphs of a series of six coregulated spots including MSN:413. In the bargraphs, the abundances of the appropriate spot (master spot number shown at the top of the panel) in each animal are shown. The five five-animal groups are in the order (left to right): high cholesterol, controls, cholestyramine, lovastatin, and lovastatin plus cholestyramine. Each bar within a group represents one experimental animal liver (one 2-D gel). Note the correlated expression of the 6 spots, especially in the two far right (most strongly induced) groups.

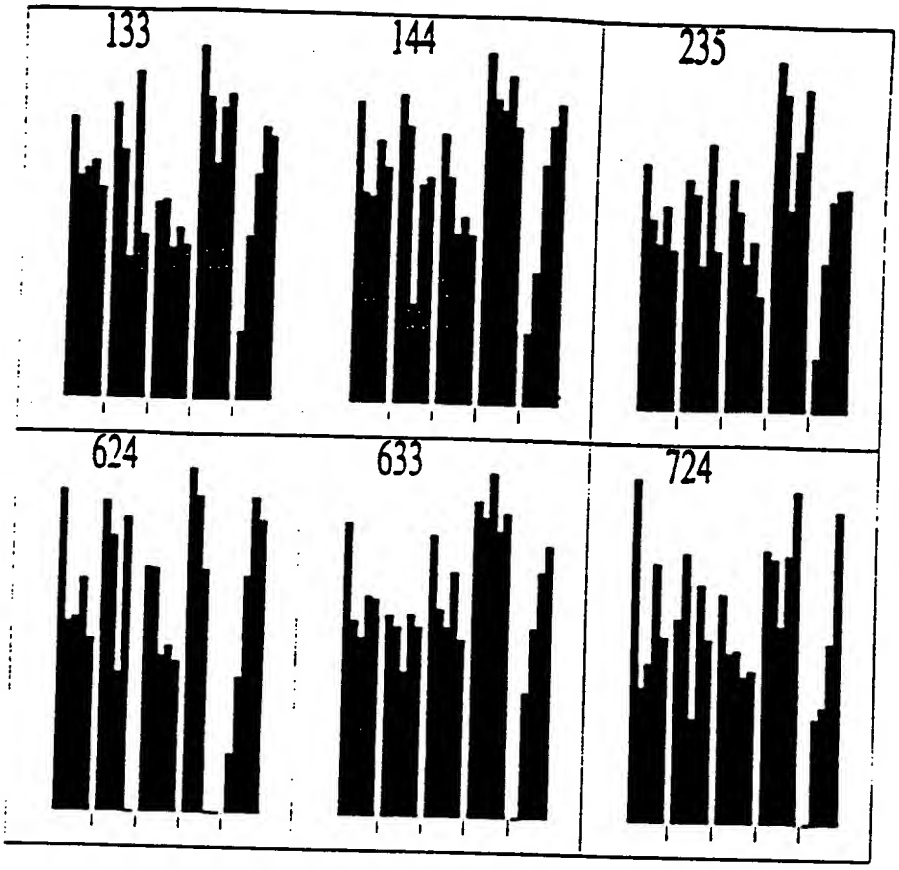


Figure 12. Data on a second coregulated group of spots, presented as in Fig. 11. The fourth experimental group (lovastatin) shows a modest induction, while the fifth group (lovastatin plus cholestyramine) does not.

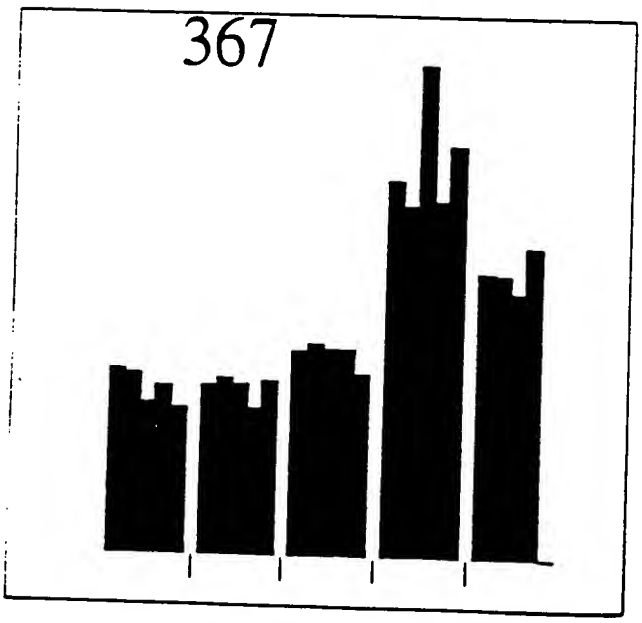


Figure 13. Data on spot MSN:367, presented as in Fig. 11. This protein shows unambiguously the anti-synergistic effect of lovastatin and cholestyramine (fifth group) as compared to lovastatin (fourth group). This response contrasts strongly with the regulation pattern seen in Fig. 11.

Addendum 2: Tables 1–4

Table 1. Master table of proteins in the rat liver database[a]

MSN	X	Y	CPKpI	SDSMW	MSN	X	Y	CPKpI	SDSMW	MSN	X	Y	CPKpI	SDSMW
3	311	434	<-35.0	63,800	95	1119	536	-9.9	53,800	174	1364	183	-6.7	162,900
5	568	263	-24.3	102,900	96	1731	756	-2.0	40,700	175	825	393	-15.7	69,300
8	812	426	-16.0	64,800	97	1033	566	-11.4	51,600	177	1582	553	-3.6	52,600
11	549	268	-25.2	101,000	98	1406	565	-6.1	51,700	178	1321	710	-7.2	43,000
15	845	520	-15.3	55,200	99	578	1149	-23.8	25,000	179	1089	615	-10.4	48,300
17	629	589	-21.6	50,000	100	2004	538	>0.0	53,700	180	1866	567	-0.5	51,600
18	906	414	-14.0	66,300	101	1106	623	-10.1	47,900	181	411	295	-32.1	91,200
19	755	298	-17.5	90,200	102	482	455	-28.5	61,300	182	804	730	-16.2	42,000
20	649	403	-20.9	67,900	103	665	830	-20.2	37,300	184	1860	896	-0.6	34,500
21	1204	448	-8.7	62,100	104	773	1182	-17.0	23,800	185	1997	1017	>0.0	29,800
22	332	434	<-35.0	63,800	105	312	1117	<-35.0	26,100	186	279	1113	<-35.0	26,300
23	787	424	-16.6	65,000	106	1769	509	-1.5	56,100	187	773	296	-17.0	90,800
24	313	417	<-35.0	66,000	107	1585	720	-3.6	42,500	188	1538	807	-4.2	38,400
25	807	516	-16.1	55,500	108	1682	807	-2.4	38,300	191	1560	674	-3.9	44,900
27	1184	524	-9.0	54,900	109	1482	593	-4.8	49,700	192	1818	687	-0.9	44,200
28	1263	446	-8.0	62,400	110	778	516	-16.9	55,500	193	1469	555	-5.0	52,400
29	743	605	-17.8	49,000	111	1728	700	-2.0	43,500	194	1380	266	-6.4	101,600
30	768	112	-17.2	348,600	113	1191	680	-8.9	44,500	195	784	632	-16.7	47,300
32	1216	417	-8.6	66,000	114	1298	185	-7.5	160,800	196	1227	1185	-8.4	23,700
33	1145	445	-9.5	62,500	115	682	907	-19.6	34,100	197	667	553	-20.1	52,600
34	1037	565	-11.3	52,400	116	1146	610	-9.5	48,700	198	2006	681	>0.0	44,500
35	863	412	-14.9	66,600	117	1548	849	-4.1	36,500	199	1711	674	-2.2	44,900
36	712	606	-18.7	48,900	118	1050	577	-11.1	50,800	200	872	424	-14.7	65,000
38	763	694	-17.3	43,800	120	1530	828	-4.3	37,400	201	292	435	<-35.0	63,700
39	304	470	<-35.0	59,800	121	838	423	-15.4	65,200	202	736	253	-18.0	107,800
41	1165	569	-9.2	51,400	122	1572	712	-3.8	42,900	203	786	829	-16.7	37,400
42	684	607	-19.6	48,800	123	23	1433	<-35.0	15,300	204	1224	589	-8.5	50,000
43	1318	589	-7.3	50,000	124	621	1474	-21.9	13,900	205	439	983	-30.9	31,100
44	1924	362	-0.1	74,600	125	1298	862	-7.5	36,000	206	1994	571	>0.0	51,300
46	1203	586	-8.7	50,200	126	872	921	-14.7	33,500	207	1895	687	-0.3	44,200
47	1391	447	-6.3	62,300	127	1000	717	-12.0	42,600	208	240	1418	<-35.0	15,800
48	309	454	<-35.0	61,500	128	1229	311	-8.4	86,100	210	1700	499	-2.3	57,000
49	605	587	-22.5	50,100	129	1422	832	-5.8	37,300	211	902	517	-14.1	55,400
50	621	535	-21.8	53,900	130	1776	499	-1.4	57,000	213	1087	684	-10.4	44,400
51	1113	522	-10.0	55,000	131	1930	757	-0.1	40,700	214	1340	668	-7.0	45,200
52	1820	499	-0.9	57,000	132	660	537	-20.4	53,800	215	1581	495	-3.5	57,300
53	725	177	-18.3	170,800	133	666	1019	-20.2	29,700	216	1585	755	-3.6	40,700
54	2001	500	>0.0	56,900	134	1271	862	-7.9	36,000	217	1159	393	-9.3	69,300
55	722	830	-18.4	37,300	135	1161	1389	-9.3	16,800	218	931	572	-13.5	51,200
56	678	533	-19.8	54,100	136	453	1063	-29.7	28,100	219	713	177	-18.7	170,500
57	1682	302	-2.5	89,000	137	1858	823	-0.6	37,700	220	1479	911	-4.9	33,900
58	1091	580	-10.3	50,600	138	1504	697	-4.6	43,700	221	965	927	-12.8	33,300
59	1171	585	-9.2	50,300	139	1488	707	-4.8	43,200	223	934	716	-13.5	42,700
60	1400	624	-6.2	47,800	140	1689	756	-2.4	40,700	225	1812	1045	-1.0	28,800
61	1853	508	-0.6	56,200	141	311	1417	<-35.0	15,800	226	821	411	-15.8	66,800
62	1888	567	-0.4	51,500	142	1366	915	-6.7	33,800	227	1586	1483	-3.6	13,600
65	735	297	-18.1	90,500	143	1429	346	-5.7	77,900	228	1065	567	-10.8	51,600
66	1263	312	-8.0	85,900	144	615	1017	-22.1	29,800	229	1577	890	-3.7	34,800
67	1252	407	-8.1	67,300	145	2006	566	>0.0	51,600	230	1458	496	-5.2	57,300
68	779	682	-16.8	43,900	146	2006	518	>0.0	55,300	232	1440	849	-5.5	36,500
69	1064	296	-10.8	90,800	147	1070	1108	-10.7	26,500	234	1692	489	-2.4	57,900
71	656	589	-20.6	50,000	148	1347	578	-6.9	50,800	235	618	1004	-22.0	30,300
72	638	545	-21.2	53,100	149	541	1481	-25.7	13,700	236	920	1138	-13.7	25,400
73	1582	583	-3.6	50,400	150	1645	760	-2.8	40,500	237	952	1008	-13.1	30,200
74	1570	556	-3.8	52,300	151	1269	236	-7.9	117,000	238	1611	541	-3.2	53,500
75	1264	621	-8.0	48,000	152	1507	911	-4.5	33,900	239	1489	720	-4.8	42,500
76	1338	564	-7.0	51,800	153	1722	448	-2.1	62,100	240	501	448	-27.7	62,100
77	1833	363	-0.8	74,400	154	932	503	-13.5	56,600	241	1820	569	-0.9	51,400
78	1767	565	-1.5	51,700	155	1031	294	-11.4	91,400	242	1357	658	-6.8	45,800
79	925	738	-13.6	41,600	156	1970	684	>0.0	44,400	243	711	1182	-18.7	23,800
80	534	698	-26.1	43,600	157	1258	183	-8.1	162,400	244	1855	621	-0.6	48,000
81	1811	363	-1.0	74,500	158	1275	417	-7.8	65,900	245	1189	474	-8.9	59,300
82	1412	681	-6.0	44,500	159	1663	820	-2.6	37,800	246	551	459	-25.1	61,000
83	1471	347	-5.0	77,500	160	1034	527	-11.4	54,600	247	1348	604	-6.9	49,100
84	1662	563	-2.7	51,800	161	1953	771	>0.0	40,000	248	460	448	-29.3	62,100
85	1596	479	-3.4	58,900	162	1020	1482	-11.6	13,700	249	1733	451	-1.9	61,800
86	1817	301	-0.9	89,100	164	1566	806	-3.8	38,400	250	1974	788	>0.0	39,200
87	516	1371	-27.0	17,400	166	1905	565	-0.2	51,700	251	808	392	-16.1	69,500
88	1589	698	-3.5	43,600	167	1340	181	-7.0	164,900	252	874	553	-14.6	52,500
89	1706	719	-2.2	42,500	168	1506	583	-4.6	50,400	253	753	848	-17.6	36,500
90	651	329	-20.8	81,700	169	1338	678	-7.0	44,700	254	995	450	-12.1	61,900
91	1415	710	-6.0	43,000	170	1969	541	>0.0	53,500	255	1690	679	-2.4	44,600
92	1773	545	-1.4	53,200	171	800	378	-16.3	71,800	256	994	1006	-12.1	30,200
93	1338	446	-7.0	62,300	172	476	958	-28.7	32,100	257	508	464	-27.4	60,400
94	1708	696	-2.2	43,700	173	919	1314	-13.7	19,300	258	1517	820	-4.4	37,800

[a] Master table of proteins in the rat liver database, showing spot master number, gel position (*x* and *y*), isoelectric point relative to CPK standards, and predicted molecular mass (from the standard curve of Fig. 8).

MSN	X	Y	CPKpl	SDSMW
259	1796	961	-1.1	31,900
260	661	1361	-20.4	17,700
261	1725	679	-2.0	44,800
262	486	1127	-28.0	25,800
263	1063	172	-10.9	177,400
265	1390	673	-6.3	45,000
266	510	437	-27.3	63,400
267	660	1038	-20.4	29,000
268	430	961	-31.0	31,800
269	1044	806	-11.2	46,800
270	2019	853	>0.0	36,300
271	857	422	-15.0	65,200
272	895	968	-14.2	31,700
274	1292	712	-7.6	42,900
275	1350	580	-6.9	49,900
276	1670	1089	-2.6	27,100
277	688	538	-19.4	53,700
278	961	718	-13.0	42,600
279	879	570	-14.5	51,300
281	1848	1084	-0.7	27,300
282	1505	525	-4.6	54,800
283	1313	1147	-7.3	25,100
284	1314	829	-7.3	37,400
285	1332	408	-7.1	67,200
286	1277	652	-7.8	46,100
288	1391	824	-6.3	37,600
289	1147	579	-9.5	50,700
290	925	511	-13.6	55,900
291	787	1476	-16.6	13,900
292	1462	818	-5.1	37,800
293	531	449	-26.3	62,000
294	860	698	-14.9	43,600
295	1162	609	-9.3	48,700
296	218	814	<-35.0	38,000
297	1377	979	-6.5	31,300
299	913	1523	-13.9	12,400
300	2012	667	>0.0	45,300
301	702	178	-19.0	169,200
302	494	1280	-28.1	20,400
303	403	1008	-32.6	30,100
304	1843	1585	-0.7	10,300
305	1049	580	-11.1	49,800
306	1608	989	-3.3	30,900
307	1219	916	-8.5	33,700
308	1627	755	-3.0	40,700
309	1524	892	-4.4	34,700
310	1769	1028	-1.5	29,400
311	1609	1451	-3.3	14,700
312	266	1408	<-35.0	16,100
313	1902	1365	-0.3	17,600
314	1316	1395	-7.3	16,600
315	1341	523	-7.0	54,900
318	1104	1053	-10.1	28,500
320	1480	1459	-4.9	14,400
321	850	603	-15.1	49,100
322	1454	1494	-5.3	13,300
323	670	626	-20.0	47,700
324	655	101	-20.6	420,500
325	1521	675	-4.4	44,800
326	1587	677	-3.6	44,700
327	1388	409	-6.3	67,000
328	448	1291	-30.0	20,100
330	1608	751	-3.3	40,900
331	1566	697	-3.8	43,700
332	531	471	-26.3	59,600
333	784	1156	-16.7	24,700
334	1059	407	-10.9	67,300
335	1593	303	-3.5	88,500
336	1616	598	-3.2	49,400
338	1854	1004	-0.6	30,300
339	1265	888	-8.0	34,900
340	581	585	-23.6	50,300
341	1497	1047	-4.7	28,700
343	1351	265	-6.8	102,200
344	1813	549	-0.9	52,800
345	1006	578	-11.9	50,800
346	1095	640	-10.3	46,800
347	625	728	-21.7	42,000
348	361	983	-35.3	31,100
349	110	1343	<-35.0	18,300
350	521	1130	-26.7	25,700
351	912	619	-13.9	48,100
352	1574	530	-3.7	54,300
353	981	912	-12.9	33,900
354	706	762	-18.9	40,400
355	1450	830	-5.3	37,300
356	1374	1152	-6.5	24,900
357	474	997	-28.7	30,600
358	798	346	-16.3	77,800
359	764	338	-17.3	79,400
360	1384	1068	-6.4	27,900
361	1713	769	-2.1	40,100
362	1161	859	-9.3	36,100
363	914	1156	-13.8	24,800
364	412	435	-32.0	63,700
365	741	486	-17.9	58,200
366	878	1503	-14.6	13,000
367	1560	935	-3.9	33,000
368	983	520	-12.4	55,200
369	434	441	-31.0	63,000
370	639	610	-21.2	48,700
371	1587	860	-3.6	36,100
372	1875	762	-0.5	40,400
373	1351	1059	-6.8	28,300
374	1506	715	-4.6	42,700
375	1823	532	-0.9	54,200
376	254	417	<-35.0	65,900
377	1409	583	-6.1	50,400
378	621	494	-21.8	57,500
379	1017	595	-11.7	49,600
381	953	598	-13.1	49,400
382	856	674	-15.0	44,900
383	1252	258	-8.1	105,300
384	1699	1518	-2.3	12,500
385	1042	493	-11.2	57,500
386	1490	583	-4.7	50,400
387	1554	603	-4.0	49,100
388	1193	404	-8.9	67,700
389	1374	902	-6.5	34,300
390	1456	969	-5.2	31,700
391	718	690	-18.5	44,000
392	1799	732	-1.1	41,900
393	1482	758	-4.8	40,600
394	1227	1461	-8.4	14,400
395	1530	577	-4.3	50,800
396	1410	755	-6.0	40,800
397	912	256	-13.9	106,400
399	1465	1063	-5.0	28,100
400	1473	450	-4.9	61,900
401	1029	1140	-11.5	25,300
403	1516	754	-4.4	40,800
404	1495	554	-4.7	52,500
405	1525	1092	-4.3	27,100
406	723	252	-18.4	108,000
409	650	663	-20.8	45,500
410	1501	478	-4.6	59,000
411	936	1057	-13.4	28,300
412	350	1120	-35.9	26,000
413	1033	538	-11.4	53,700
415	737	425	-18.0	64,900
416	1578	606	-3.7	48,900
417	646	496	-21.0	57,300
418	1695	482	-2.3	58,600
419	725	770	-18.3	40,000
420	1289	1041	-7.7	28,900
421	1171	912	-9.1	33,900
422	599	162	-22.8	193,700
423	929	856	-13.6	36,200
424	739	625	-17.9	47,700
425	1490	965	-4.7	31,800
426	1296	704	-7.6	43,200
427	810	843	-16.0	36,800
428	1565	303	-3.9	88,700
429	1259	847	-8.0	36,800
430	1253	562	-8.1	51,800
431	734	1426	-18.1	15,900
432	483	433	-28.5	63,900
434	518	1041	-26.9	28,900
435	1020	1170	-11.6	24,300
436	1122	196	-9.8	147,800
437	1870	673	-0.5	45,000
438	435	1102	-31.0	26,700
439	86	847	<-35.0	36,800
440	1740	544	-1.8	53,200
441	599	1571	-22.8	10,800
443	743	335	-17.8	80,100
446	801	668	-16.2	45,200
447	1050	926	-11.1	33,300
448	1245	1298	-8.2	19,800
449	1576	1516	-3.7	12,600
450	1818	1021	-0.9	29,600
451	1094	440	-10.3	63,100
452	1945	802	>0.0	38,600
453	1652	894	-2.8	34,600
454	1403	500	-6.1	56,800
456	1394	718	-6.3	42,600
457	905	436	-14.0	63,500
459	1038	581	-11.3	50,500
460	1598	294	-3.4	91,400
461	1528	863	-4.3	35,900
462	1098	1137	-10.2	25,400
463	849	1125	-15.2	25,800
464	1814	1072	-0.9	27,800
465	1388	481	-6.3	58,700
466	1194	1084	-8.9	27,300
468	577	467	-23.9	60,100
469	1140	888	-9.6	34,900
470	1797	524	-1.1	54,800
471	1293	1133	-7.6	25,500
472	618	655	-21.9	46,000
473	2009	299	>0.0	89,900
474	1205	215	-8.7	131,300
475	1035	788	-11.4	39,200
476	160	155	<-35.0	207,600
477	469	1370	-28.9	17,400
478	599	662	-22.8	45,800
479	1009	540	-11.8	53,900
480	1216	235	-8.6	117,400
482	816	346	-15.9	77,800
483	693	673	-19.3	44,900
485	1608	1013	-3.3	30,000
486	478	599	-28.6	49,300
487	1025	607	-11.5	48,800
488	1045	1186	-11.2	23,700
489	1609	301	-3.3	89,200
490	775	1289	-17.0	20,100
491	692	178	-19.3	169,300
492	1100	964	-10.2	31,800
493	1760	776	-1.6	39,700
494	882	247	-14.5	110,700
495	470	1258	-28.9	21,200
496	494	1436	-28.1	15,200
497	980	852	-12.5	36,400
499	1414	546	-6.0	53,100
500	1234	1072	-8.3	27,800
501	1246	659	-8.2	45,700
502	824	792	-15.7	39,000
503	1246	1134	-8.2	25,500
504	1115	1407	-9.9	16,200
505	1189	391	-8.9	69,700
506	1578	402	-3.7	68,000
507	787	250	-16.6	109,000
508	979	552	-12.5	52,800
509	1153	619	-9.4	48,100
510	1730	1006	-2.0	30,200

MSN	X	Y	CPKal	SDSMW
511	809	484	-16.0	58,400
512	1099	533	-10.2	54,100
513	1696	1034	-2.3	29,200
514	948	636	-13.2	47,100
515	481	543	-28.5	53,400
516	1334	1044	-7.1	28,800
517	868	1021	-14.8	29,700
518	798	779	-16.3	39,600
519	822	670	-15.7	45,100
520	632	165	-21.5	189,000
521	1332	830	-7.1	37,300
522	603	1104	-22.6	26,600
523	1190	309	-8.9	86,800
524	479	1226	-28.6	22,300
525	768	1066	-17.2	28,000
526	747	1016	-17.7	29,800
527	1170	231	-9.2	119,600
528	1502	542	-4.6	53,400
530	1728	620	-2.0	48,000
532	507	1011	-27.4	30,000
533	870	489	-14.7	57,900
534	1347	1085	-6.9	27,300
535	1513	346	-4.5	77,800
536	308	654	<-35.0	46,000
538	1851	689	-0.7	44,100
539	1463	982	-5.1	31,100
540	909	561	-13.9	52,000
541	625	289	-21.7	93,100
542	1164	198	-9.2	146,200
543	803	655	-16.2	45,900
544	1259	1143	-8.0	25,200
545	856	1526	-15.0	12,200
546	803	1071	-16.2	27,800
547	1162	274	-9.3	98,400
548	128	1321	<-35.0	19,000
549	1355	1122	-6.8	25,900
550	595	866	-23.0	35,800
552	1369	494	-6.6	57,500
553	992	405	-12.2	67,600
555	1125	410	-9.8	66,900
556	705	975	-18.9	31,400
557	1477	1030	-4.9	29,300
558	980	583	-12.5	50,400
559	700	1109	-19.1	26,400
560	1028	621	-11.5	48,000
562	898	794	-14.1	38,900
564	789	1446	-16.6	14,900
565	777	766	-16.9	40,200
566	980	328	-12.5	81,900
567	1519	611	-4.4	48,600
568	1212	661	-8.6	45,600
570	760	594	-17.4	49,700
571	618	956	-21.9	32,100
573	1142	771	-9.6	40,000
574	532	787	-26.2	39,300
575	771	250	-17.1	109,200
576	1068	534	-10.8	54,100
577	822	734	-15.7	41,800
578	914	754	-13.8	40,800
579	1064	794	-10.8	38,900
580	1524	714	-4.4	42,800
581	1392	783	-6.3	39,400
582	982	686	-12.4	44,200
584	1487	672	-4.8	45,000
585	758	731	-17.4	41,900
586	687	1152	-19.5	24,900
587	930	523	-13.5	55,000
588	1888	774	-0.4	39,900
589	642	485	-21.1	58,300
590	1317	519	-7.3	55,300
591	65	1548	<-35.0	11,500
592	1014	614	-11.7	48,400
593	732	176	-18.1	172,300
594	1627	478	-3.0	59,000
595	1009	1426	-11.8	15,500
596	619	269	-21.9	100,500
597	1176	461	-9.1	60,700
598	1465	1044	-5.0	28,800
599	741	1188	-17.9	23,600
600	907	402	-14.0	68,000
601	687	658	-19.5	45,800
602	712	1138	-18.7	25,400
603	898	181	-14.1	165,200
604	783	1461	-16.7	14,400
605	736	223	-18.0	125,300
606	629	273	-21.6	98,700
607	1064	286	-10.8	94,000
608	883	503	-14.5	56,700
609	2012	610	>0.0	48,700
610	1255	903	-8.1	34,200
612	1103	391	-10.1	69,600
613	778	265	-16.9	102,000
614	824	518	-15.7	55,400
615	1095	195	-10.3	149,100
616	1759	478	-1.6	59,000
617	994	372	-12.1	72,900
618	751	374	-17.6	72,400
619	1429	518	-5.7	55,300
620	1050	520	-11.1	55,200
621	923	1105	-13.7	26,600
622	1462	622	-5.1	47,900
623	759	225	-17.4	124,000
624	758	1038	-17.4	29,000
625	1438	606	-5.5	48,900
626	1096	1089	-10.2	27,200
627	942	548	-13.3	53,000
628	809	621	-16.0	48,000
629	899	979	-14.1	31,300
630	1135	1321	-9.6	19,100
631	979	615	-12.5	48,300
632	1542	1076	-4.1	27,600
633	1345	814	-6.9	38,000
634	409	950	-32.2	32,400
635	1165	704	-9.2	43,300
636	774	604	-17.0	49,000
637	1263	524	-8.0	54,800
638	952	411	-13.1	66,700
639	1717	575	-2.1	51,000
640	994	292	-12.1	92,000
641	165	1224	<-35.0	22,400
642	803	251	-16.2	108,900
643	719	296	-18.5	90,700
644	1100	294	-10.2	91,400
645	534	1263	-26.1	21,000
646	1153	1038	-9.4	29,000
648	1246	204	-8.2	140,000
649	14	1406	<-35.0	16,200
650	1713	1049	-2.1	28,600
651	1986	1183	>0.0	23,800
652	1378	816	-6.5	38,000
653	1442	1165	-5.5	24,400
654	650	806	-20.8	38,400
655	1111	551	-10.0	52,700
656	1095	861	-10.3	36,000
657	1524	540	-4.4	53,600
658	1777	860	-1.4	36,000
659	391	584	-33.4	50,400
660	977	565	-12.5	51,700
661	658	166	-20.5	187,500
662	732	312	-18.1	86,100
663	1787	567	-1.2	51,500
664	888	268	-14.4	100,900
665	889	775	-14.3	39,800
666	715	221	-18.6	126,300
667	781	227	-16.8	122,400
668	646	165	-21.0	189,100
669	1116	353	-9.9	76,300
670	1382	643	-6.4	46,600
671	547	789	-25.3	39,200
673	984	746	-12.4	41,200
674	1661	448	-2.7	62,100
675	1523	562	-4.4	51,900
676	708	642	-18.8	46,700
677	919	615	-13.7	48,300
678	1085	551	-10.5	52,700
679	600	923	-22.7	33,400
680	1237	1004	-8.3	30,300
681	1103	283	-10.1	95,100
682	1406	477	-6.1	59,100
683	1596	249	-3.4	109,800
684	555	699	-24.8	43,500
685	1167	1313	-9.2	19,300
686	1932	790	0.0	39,100
687	1545	619	-4.1	48,100
688	1456	764	-5.2	40,300
689	1011	953	-11.8	32,300
690	1995	270	>0.0	100,200
691	812	886	-16.0	34,900
692	1154	1461	-9.4	14,400
693	1993	819	>0.0	37,800
694	1628	656	-3.0	45,900
695	928	254	-13.6	107,000
696	1854	715	-0.6	42,700
697	1997	345	>0.0	78,000
698	957	563	-13.0	51,800
699	1540	730	-4.2	42,000
702	577	900	-23.8	34,400
703	1610	562	-3.2	51,900
705	1278	571	-7.8	51,200
706	1841	704	-0.7	43,300
707	1018	1386	-11.7	16,900
709	1074	1145	-10.7	25,100
710	293	889	<-35.0	34,800
712	720	412	-18.5	66,600
713	1386	841	-6.4	36,800
714	1328	263	-7.1	103,100
715	698	433	-19.1	63,900
716	701	481	-19.0	58,700
717	1875	699	-0.5	43,600
718	575	702	-23.9	43,400
719	1216	204	-8.6	140,400
721	1069	464	-10.8	60,400
722	1272	506	-7.9	56,400
723	958	822	-13.0	37,700
724	763	395	-17.3	69,100
725	720	916	-18.5	33,700
726	1476	415	-4.9	66,200
727	1846	473	-0.7	59,400
728	510	783	-27.3	39,400
729	1217	1126	-8.6	25,800
730	1858	724	-0.6	42,300
731	665	765	-20.2	40,300
733	1321	312	-7.2	85,900
734	719	427	-18.5	64,600
735	1101	473	-10.2	59,500
736	1359	569	-6.7	51,400
738	696	220	-19.2	127,600
739	687	409	-19.5	67,000
740	1205	256	-8.7	106,200
741	995	563	-12.1	51,900
742	898	596	-14.1	49,500
743	881	181	-14.5	165,900
744	1951	686	>0.0	44,200
745	726	168	-18.3	183,600
746	999	643	-12.0	46,600
748	182	1503	<-35.0	13,000
749	2005	649	>0.0	46,300
750	1448	575	-5.4	51,000
751	792	266	-16.5	101,900
752	469	296	-28.9	90,600
754	664	254	-20.3	107,000
755	1195	184	-8.8	161,000
756	1821	1113	-0.9	26,300
757	909	246	-13.9	111,000
760	790	133	-16.5	264,900

MSN	X	Y	CPKpl	SDSMW
761	1399	733	-6.2	41,800
763	1416	1085	-5.9	27,300
764	2020	569	>0.0	51,400
765	651	475	-20.8	59,300
766	1052	1149	-11.1	25,000
767	1968	468	>0.0	59,900
768	1330	685	-7.1	44,300
769	1970	613	>0.0	48,500
770	857	617	-15.0	48,200
771	1337	974	-7.0	31,500
773	1576	502	-3.7	56,700
775	969	824	-12.8	37,600
776	1438	708	-5.5	43,100
777	1539	458	-4.2	61,000
778	850	434	-15.1	63,800
779	700	411	-19.1	66,800
780	1052	1136	-11.1	25,500
784	1413	529	-6.0	54,400
785	1364	885	-6.7	35,000
786	1822	835	-0.9	37,100
787	893	392	-14.3	69,500
790	616	882	-22.0	35,100
791	451	1429	-29.8	15,400
792	777	377	-16.9	72,000
793	1536	1543	-4.2	11,700
794	1461	807	-5.1	38,300
796	388	546	-33.6	53,100
797	1126	212	-9.8	133,700
798	933	437	-13.5	63,400
799	1420	593	-5.9	49,800
800	1758	279	-1.6	96,500
801	624	865	-21.7	35,800
802	898	547	-14.2	53,000
803	1775	1468	-1.4	14,200
804	573	196	-24.0	148,400
805	203	494	<-35.0	57,400
806	980	1039	-12.5	29,000
807	902	308	-14.1	87,200
808	625	827	-21.7	37,500
809	1851	1015	-0.7	29,900
810	440	573	-30.9	51,100
811	1358	249	-6.8	109,700
812	851	393	-15.1	69,400
813	745	1246	-17.8	21,600
814	2028	810	>0.0	38,200
815	1086	645	-10.4	46,500
816	629	313	-21.6	85,700
817	1376	1177	-6.5	24,000
818	1771	790	-1.4	39,100
819	1045	263	-11.2	103,100
820	984	362	-12.4	74,600
821	1712	279	-2.2	96,700
822	1256	205	-8.1	139,200
823	1517	654	-4.4	46,000
824	1442	449	-5.5	62,000
825	1240	513	-8.3	55,800
826	1309	1014	-7.4	29,900
827	2012	708	>0.0	43,100
828	937	1405	-13.4	16,200
830	1342	756	-7.0	40,700
831	562	826	-24.5	37,500
832	1073	1039	-10.7	29,000
833	481	820	-28.5	37,800
834	501	581	-27.8	50,500
837	751	748	-17.6	41,100
838	635	833	-21.3	37,200
839	1494	459	-4.7	60,900
840	1952	301	>0.0	89,300
841	1585	1080	-3.6	27,500
842	571	1312	-24.1	19,400
843	1325	649	-7.2	46,300
844	1727	301	-2.0	89,200
845	630	679	-21.5	44,600
846	2016	905	>0.0	34,200
847	673	1200	-19.9	23,200
848	1863	271	-0.6	99,500
849	1166	523	-9.2	54,900
850	1535	1024	-4.2	29,600
851	1035	826	-11.4	37,500
852	834	542	-15.5	53,400
855	499	220	-27.8	127,100
856	1063	194	-10.9	150,500
857	887	890	-14.4	34,800
858	1448	639	-5.4	46,900
859	706	311	-18.9	86,200
860	1070	1066	-10.7	28,000
861	472	347	-28.8	77,600
862	674	480	-19.9	58,800
864	1307	499	-7.4	57,000
865	645	887	-21.0	34,900
866	827	1004	-15.6	30,300
868	685	494	-19.5	57,400
869	1807	402	-1.0	68,000
870	1323	783	-7.2	39,400
871	1228	1031	-8.4	29,300
872	1904	346	-0.3	77,700
873	556	647	-24.8	46,400
874	1540	756	-4.2	40,700
875	1566	777	-3.8	39,700
876	1198	351	-8.8	76,800
877	1076	720	-10.6	42,500
878	1161	1111	-9.3	26,400
879	647	757	-20.9	40,700
880	1756	594	-1.6	49,700
881	1543	278	-4.1	97,100
883	1432	890	-5.7	34,800
884	922	689	-13.7	44,100
885	1103	414	-10.1	66,400
886	1501	607	-4.6	48,900
887	798	1103	-16.3	26,600
888	636	634	-21.3	47,200
889	951	759	-13.1	40,600
890	717	548	-18.6	52,900
891	1123	229	-9.8	121,200
892	891	413	-14.3	66,400
894	1245	234	-8.2	117,800
895	1962	346	>0.0	77,700
896	1322	626	-7.2	47,700
897	420	570	-31.4	51,300
898	662	428	-20.3	64,500
899	845	243	-15.3	113,000
900	624	703	-21.7	43,400
901	931	1094	-13.5	27,000
903	799	229	-16.3	121,000
904	765	520	-17.2	55,200
905	775	889	-17.0	34,800
907	888	824	-14.4	37,600
908	828	1303	-15.6	19,700
910	681	1544	-19.7	11,700
911	1544	301	-4.1	89,100
913	1606	387	-3.3	70,400
914	1237	688	-8.3	44,100
916	1442	749	-5.5	41,100
917	1260	367	-8.0	73,700
919	764	1541	-17.3	11,700
920	1133	1123	-9.7	25,900
921	1123	380	-9.8	71,500
923	829	242	-15.6	113,200
924	1131	318	-9.7	84,300
925	1441	874	-5.5	35,400
926	679	219	-19.7	128,200
927	1487	1191	-4.8	23,500
928	1082	775	-10.5	39,800
929	1231	816	-8.4	38,000
931	1609	670	-3.3	45,100
932	810	900	-16.0	34,400
933	965	520	-12.8	55,100
934	947	462	-13.2	60,600
936	865	843	-14.8	36,800
937	1421	1056	-5.9	28,400
939	1197	827	-8.8	37,500
941	1765	885	-1.5	35,000
942	602	472	-22.7	59,600
943	312	498	<-35.0	57,100
944	993	491	-12.1	57,700
945	1300	269	-7.5	100,300
946	630	423	-21.6	65,100
947	187	736	<-35.0	41,600
948	1380	344	-6.5	78,200
949	1766	665	-1.5	45,400
950	1038	193	-11.3	151,000
951	860	152	-14.9	213,000
952	957	701	-13.0	43,400
954	503	547	-27.6	53,000
955	1938	712	>0.0	42,900
957	1010	816	-11.8	37,900
959	768	174	-17.2	174,900
960	596	419	-23.0	65,700
961	557	409	-24.8	67,100
962	887	320	-14.4	83,900
963	564	334	-24.5	80,500
964	969	1155	-12.8	24,800
965	671	255	-20.0	106,600
966	1204	798	-8.7	38,700
967	910	154	-13.9	210,300
968	609	1048	-22.3	28,700
969	1285	206	-7.7	138,900
970	822	232	-15.8	119,300
971	976	437	-12.6	63,400
972	403	567	-32.6	51,600
974	279	495	<-35.0	57,400
975	844	981	-15.3	31,200
976	1124	295	-9.8	91,100
977	994	664	-12.1	45,400
978	1612	642	-3.2	46,700
979	749	1141	-17.7	25,300
980	1064	642	-10.8	46,700
981	1197	911	-8.8	33,900
983	1762	1508	-1.6	12,800
984	1344	317	-6.9	84,700
985	1024	1105	-11.5	26,600
987	739	1159	-17.9	24,600
988	816	555	-15.9	52,400
990	785	361	-16.7	74,900
991	1159	317	-9.3	84,500
992	1090	928	-10.4	33,300
993	1030	701	-11.5	43,400
994	847	811	-15.2	38,200
995	902	461	-14.1	60,700
996	888	847	-14.4	36,600
997	1815	579	-0.9	50,700
998	1205	504	-8.7	56,500
999	617	289	-22.0	93,100
1000	968	290	-12.8	92,700
1001	970	771	-12.7	40,000
1002	1736	478	-1.9	58,900
1003	643	1184	-21.1	23,700
1006	822	487	-15.8	58,100
1007	875	279	-14.6	96,400
1009	291	644	<-35.0	46,600
1010	1386	745	-6.4	41,200
1011	459	541	-29.4	53,500
1012	679	661	-19.7	45,600
1013	1818	1128	-0.9	25,600
1014	1032	634	-11.4	47,200
1015	1629	994	-3.0	30,700
1016	1311	1134	-7.4	25,500
1017	1722	424	-2.0	65,000
1018	1015	743	-11.7	41,300
1020	1574	1219	-3.7	22,900
1021	781	484	-16.8	58,400
1022	1129	83	-9.7	591,300
1023	812	317	-15.9	84,600
1024	785	446	-16.7	62,400
1025	1290	739	-7.7	41,500

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MSN	X	Y	CPKoi	SDSMW
1026	405	552	-32.3	52,600
1027	1298	848	-7.5	36,500
1028	856	547	-15.0	53,000
1030	1284	226	-7.7	123,200
1031	986	822	-12.3	37,700
1032	1547	403	-4.1	67,900
1033	1381	551	-6.4	52,700
1034	1525	496	-4.3	57,200
1035	1128	645	-9.7	46,500
1036	1226	274	-8.5	98,300
1039	1761	262	-1.6	103,600
1040	541	839	-25.7	36,900
1041	818	910	-15.8	34,000
1044	1036	485	-11.3	58,300
1045	1439	407	-5.5	67,300
1047	1540	250	-4.2	109,200
1048	1576	635	-3.7	47,100
1049	1089	411	-10.4	66,700
1050	949	1040	-13.2	28,900
1051	426	818	-31.1	37,800
1052	1583	1385	-3.6	16,900
1053	779	1092	-16.8	27,000
1054	1613	620	-3.2	48,000
1055	1380	377	-6.5	72,000
1056	284	663	<-35.0	45,500
1058	1261	746	-8.0	41,200
1060	393	605	-33.3	49,000
1061	1817	645	-0.9	46,600
1062	1245	746	-8.2	41,200
1064	1258	792	-8.1	39,000
1065	705	934	-18.9	33,000
1066	1181	734	-9.0	41,800
1067	529	658	-26.3	45,800
1068	508	696	-27.4	43,700
1069	1898	604	-0.3	49,100
1071	873	609	-14.7	48,700
1073	1768	1128	-1.5	25,800
1075	836	773	-15.4	39,900
1076	1863	861	-0.6	36,000
1078	826	566	-15.7	51,600
1081	971	483	-12.7	58,500
1083	1697	202	-2.3	142,300
1085	1157	794	-9.4	38,900
1090	620	910	-21.9	34,000
1092	1867	597	-0.5	49,500
1093	2019	894	>0.0	34,600
1094	1546	538	-4.1	53,700
1095	1545	477	-4.1	59,100
1098	61	935	<-35.0	33,000
1099	1954	237	>0.0	116,000
1101	588	1048	-23.3	28,600
1102	1050	667	-11.1	45,200
1103	457	797	-29.5	38,800
105	1884	532	-0.4	54,200
106	1714	649	-2.1	46,300
107	1717	546	-2.1	53,100
108	1976	722	>0.0	42,400
1111	547	1066	-25.3	28,000
1112	1348	621	-6.9	48,000
1115	1385	762	-6.4	40,400
116	1078	816	-10.6	38,000
117	975	787	-12.6	39,300
118	1202	933	-8.7	33,100
119	1022	1076	-11.6	27,600
120	1905	616	-0.3	48,300
121	1512	1301	-4.5	19,700
122	1114	677	-9.9	44,700
123	1464	452	-5.1	61,700
125	1048	857	-11.1	36,200
126	1122	802	-9.8	38,600
128	1722	892	-2.1	34,700
133	1098	825	-10.2	37,500
139	1830	569	-0.8	51,400
147	764	1182	-17.3	23,800
148	1968	724	>0.0	42,300

MSN	X	Y	CPKoi	SDSMW
1153	921	1158	-13.7	24,700
1154	1594	864	-3.5	35,900
1161	637	400	-21.3	68,400
1162	623	397	-21.8	68,800
1163	665	397	-20.2	68,700
1168	564	528	-24.4	54,500
1170	562	529	-25.0	54,500
1171	538	524	-25.9	54,800
1172	545	514	-25.5	55,700
1174	1099	522	-10.2	55,000
1176	1304	586	-7.5	50,200
1177	1366	539	-6.6	53,700
1178	1608	702	-3.3	43,400
1179	1485	224	-4.8	124,900
1180	1459	224	-5.2	124,900
1181	1431	223	-5.7	125,100
1182	1407	223	-6.1	125,200
1183	1383	224	-6.4	124,700
1184	1454	182	-5.3	164,400
1185	1422	183	-5.8	162,600
1186	1394	182	-6.3	164,300
1189	1171	214	-9.2	131,800
1190	1457	286	-5.2	94,200
1191	686	1114	-19.5	26,200
1192	265	883	<-35.0	34,700
1193	403	1292	-32.6	20,000
1194	344	1275	<-35.0	20,600
1195	505	1311	-27.6	19,400
1196	572	1293	-24.1	20,000
1197	639	1502	-21.2	13,000
1198	637	1402	-21.3	16,300
1199	614	1407	-22.1	16,200
1200	637	1431	-21.3	15,400
1201	1095	1394	-10.3	16,600
1202	1719	1545	-2.1	11,600
1203	791	668	-16.5	45,200
1204	964	1021	-12.9	29,700
1205	313	195	<-35.0	148,700
1208	306	194	<-35.0	149,800
1209	320	197	<-35.0	147,400
1210	326	197	<-35.0	146,600
1211	394	294	-33.2	91,400
1212	402	294	-32.7	91,200
1214	386	294	-33.7	91,400
1215	641	329	-21.2	81,600
1216	660	329	-20.4	81,600
1217	914	266	-13.8	101,800
1218	873	245	-14.7	112,000
1219	970	372	-12.7	72,900
1220	1021	298	-11.6	90,100
1221	1392	205	-6.3	139,500
1222	1354	203	-6.8	141,800
1223	1362	205	-6.7	139,500
1224	673	540	-19.9	53,600
1225	614	542	-22.1	53,400
1226	603	539	-22.6	53,600
1227	696	623	-19.2	47,800
1228	707	628	-18.9	47,500
1229	475	447	-28.7	62,300
1230	466	1282	-29.0	20,400
1231	759	1461	-17.4	14,400
1232	1324	1170	-7.2	24,200
1233	1583	1005	-3.6	30,300
1234	1865	809	-0.6	38,200
1235	1812	817	-1.0	37,900
1236	1411	703	-6.0	43,400
1237	1392	682	-6.3	44,500
1238	794	410	-16.4	66,900
1239	769	407	-17.1	67,300
1240	740	406	-17.9	67,500
1241	743	511	-17.8	55,900
1242	713	510	-18.7	56,000
1243	682	509	-19.6	56,100
1244	663	504	-20.3	56,500
1245	565	582	-24.4	50,500

MSN	X	Y	CPKoi	SDSMW
1246	547	577	-25.3	50,800
1247	530	576	-26.3	50,900
1249	516	572	-27.0	51,200
1250	973	536	-12.7	53,900
1251	607	532	-22.4	54,200
1252	665	529	-20.2	54,400
1253	899	766	-14.1	40,200
1254	1311	746	-7.4	41,200
1255	1300	761	-7.5	40,400
1257	1938	712	0.0	42,900
1258	1806	718	-1.0	42,600
1259	1727	715	-2.0	42,700
1260	1629	713	-3.0	42,800
1261	1555	717	-4.0	42,600
1262	1468	717	-5.0	42,600
1263	1413	722	-6.0	42,400
1264	1340	717	-7.0	42,600
1265	1263	717	-8.0	42,600
1266	1182	720	-9.0	42,500
1267	1110	717	-10.0	42,600
1268	1055	717	-11.0	42,600
1269	999	717	-12.0	42,600
1270	959	715	-13.0	42,700
1271	905	712	-14.0	42,900
1272	857	714	-15.0	42,800
1273	810	705	-16.0	43,300
1274	774	711	-17.0	42,900
1277	737	708	-18.0	43,100
1278	702	711	-19.0	42,900
1279	671	710	-20.0	43,000
1280	645	710	-21.0	43,000
1281	617	707	-22.0	43,100
1282	595	704	-23.0	43,300
1283	573	700	-24.0	43,500
1284	552	695	-25.0	43,700
1285	536	694	-26.0	43,800
1286	515	687	-27.0	44,200
1287	496	683	-28.0	44,400
1288	467	669	-29.0	45,200
1289	447	667	-30.9	45,300
1290	427	655	-31.0	45,900
1291	412	655	-32.0	45,900
1292	397	652	-33.0	46,100
1293	381	654	-34.0	46,000
1294	365	653	-35.0	46,100
1295	348	653	<-35.0	46,100

Table 2. Table of some identified proteins

POP name	Protein name	MSN's	Basis for identification
IDS:3_ALPHA_HDDH	3-α-hydroxysteroid-dihydrodiol-dehydrogenase, an enzyme of steroid metabolism	137, 159	Pure protein and antibody provided by Dr. T.M. Penning, Department of Pharmacology, School of Medicine, University of Pennsylvania.
IDS:ACTIN_BETA	β cellular actin, a cytoskeletal protein	38	Homologous position with respect to other mammalian systems
IDS:ACTIN_GAMMA	γ cellular actin, a cytoskeletal protein	68	Homologous position with respect to other mammalian systems
IDS:ALBUMIN	Serum albumin, mature form.	21, 28, 33	Predominance in rat plasma
IDS:APO_A-I	Apo A-I plasma lipoprotein, mature form (tentative).	236, 463	Presence in rat plasma, regulation by some lipid-lowering drugs
IDS:CALMODULIN	Calmodulin, an acidic cytosolic calcium-binding protein	123, 649	Homologous position with respect to other mammalian systems
IDS:CATALASE	Catalase (peroxisomal)	54, 61, 106	Presence in purified peroxisomes, similarity in position to mouse catalase
IDS:CPKSPOTS	Spots contributed by the CPK charge standards (not rat liver proteins)	1257 - 1295	
IDS:CPS	Carbamoyl phosphate synthase	114, 157, 167, 174, 1184, 1185, 1186, 1222	Pure protein provided by Dr. Margaret Marshall, Department of Pharmacology, Medical School, University of Wisconsin - Madison.
IDS:CYTOCHROME_B5	Cytochrome b5	87, 477	Pure protein provided by Dr. Andrew Parkinson, Department of Pharmacology, Toxicology and Therapeutics, University of Kansas Medical Center
IDS:FABP-L	Liver fatty-acid binding protein	227	Pure protein provided by Dr. Nathan Bass, Department of Medicine, University of California School of Medicine, San Francisco
IDS:HMG-COA_SYNTHASE	Cytosolic HMG-CoA Synthase	133, 144, 235, 413	Antibody provided by Dr. Michael Greenspan, Merck Sharp & Dohme Research Laboratories, Rahway, NJ
IDS:LAMIN_B	Lamin B, a nuclear protein	415, 734	Homologous position with respect to other mammalian systems
IDS:MITCON:1	Mitcon:1 (F1 ATPase β subunit), a mitochondrial inner membrane	17, 49, 71, 340, 1245, 1246, 1247, 1249	Homologous position with respect to other mammalian systems, presence in mitochondria
IDS:MITCON:2	Mitcon:2, a mitochondrial matrix stress protein equivalent to E.	15, 25, 110, 1241, 1242, 1243, 1244	Homologous position with respect to other mammalian systems, presence in mitochondria
IDS:MITCON:3	Mitcon:3, a mitochondrial matrix stress protein, likely analog of	18, 35, 226, 600, 1238, 1239, 1240	Homologous position with respect to other mammalian systems, presence in mitochondria
IDS:NADPH_P450_RED	NADPH cytochrome P-450 reductase, frequently co-induced with P-450's	175, 251, 812	Pure protein provided by Dr. Andrew Parkinson, Department of Pharmacology, Toxicology and Therapeutics, University of Kansas Medical Center
IDS:PDI	Protein disulphide isomerase 1	168, 1170, 1171, 1172	Sequence information obtained by R.M. Van Frank, Lilly Research Laboratories, Indianapolis
IDS:PLASMA_PROTEINS	Rat plasma proteins observed in liver	21, 28, 33, 44, 72, 102, 115, 197, 236, 246, 248, 257, 293, 332, 347, 364, 369, 419, 432, 463, 468, 518, 562, 605, 623, 666, 667, 725, 738, 790, 865, 903, 926	Plasma coelectrophoresis studies
IDS:PRO-ALBUMIN	Serum albumin precursor	47, 93	Relative position to mature albumin, presence in microsomes
IDS:PYRCARBOX	Pyruvate carboxylase	179, 1180, 1181, 1182, 1183	Pavlica, R.J., et al., BBA (1990) *1022* 115-125.
IDS:SOD	Superoxide dismutase	135	Sequence information obtained by R.M. Van Frank, Lilly Research Laboratories, Indianapolis
IDS:TUBULIN_ALPHA	α tubulin, a cytoskeletal protein	56, 132, 1224, 1252	Homologous position with respect to other mammalian systems
IDS:TUBULIN_BETA	β tubulin, a cytoskeletal protein	50, 1225, 1226, 1251	Homologous position with respect to other mammalian systems

e 3. Computed pI's of two sets of carbamylated protein standards: Rabbit muscle CPK and human hemoglobin (Hb)

Protein Name	PIR Name	#ASP 3.9	#GLU 4.1	#HIS 6.0	#LYS 10.8	#ARG 12.5	NH2- 7.0	Calc pI	Real CPK
Rabbit muscle CPK	KIRBCM	28	27	17	34	18	1	6.84	0.0
		28	27	17	33	18	1	6.67	-1
		28	27	17	32	18	1	6.54	-2
		28	27	17	31	18	1	6.42	-3
		28	27	17	30	18	1	6.31	-4
		28	27	17	29	18	1	6.21	-5
		28	27	17	28	18	1	6.12	-6
		28	27	17	27	18	1	6.03	-7
		28	27	17	26	18	1	5.94	-8
		28	27	17	25	18	1	5.85	-9
		28	27	17	24	18	1	5.76	-10
		28	27	17	23	18	1	5.67	-11
		28	27	17	22	18	1	5.58	-12
		28	27	17	21	18	1	5.48	-13
		28	27	17	20	18	1	5.39	-14
		28	27	17	19	18	1	5.29	-15
		28	27	17	18	18	1	5.20	-16
		28	27	17	17	18	1	5.12	-17
		28	27	17	16	18	1	5.04	-18
		28	27	17	15	18	1	4.96	-19
		28	27	17	14	18	1	4.89	-20
		28	27	17	13	18	1	4.83	-21
		28	27	17	12	18	1	4.77	-22
		28	27	17	11	18	1	4.71	-23
		28	27	17	10	18	1	4.66	-24
		28	27	17	9	18	1	4.61	-25
		28	27	17	8	18	1	4.56	-26
		28	27	17	7	18	1	4.52	-27
		28	27	17	6	18	1	4.48	-28
		28	27	17	5	18	1	4.44	-29
		28	27	17	4	18	1	4.40	-30
		28	27	17	3	18	1	4.36	-31
		28	27	17	2	18	1	4.32	-32
		28	27	17	1	18	1	4.29	-33
		28	27	17	0	18	1	4.25	-34
		28	27	17	0	18	0	4.22	-35
Hb-beta, human	HBHU	7	8	9	11	3	1	7.18	
		7	8	9	10	3	1	6.79	
		7	8	9	9	3	1	6.53	-1.8
		7	8	9	8	3	1	6.32	-3.2
		7	8	9	7	3	1	6.13	-5.3
		7	8	9	6	3	1	5.96	-7.2
		7	8	9	5	3	1	5.78	-10.0
		7	8	9	4	3	1	5.59	-12.3
		7	8	9	3	3	1	5.37	-15.5
		7	8	9	2	3	1	5.14	-18.0
		7	8	9	1	3	1	4.91	-21.0
		7	8	9	0	3	1	4.71	-25.5
		7	8	9	0	3	0	4.54	-27.2

Table 4. Computed pI's of some known proteins related to measured CPK pI's

	Protein Name	PIR Name	#ASP 3.9	#GLU 4.1	#HIS 6.0	#LYS 10.8	#ARG 12.5	Calc pI	Real CPK
0	Creatine phospho kinase (CPK), rabbit muscle	KIRBCM	28	27	17	34	18	6.84	0.0
1	Fatty acid-binding protein, rat hepatic	FZRTL	5	13	2	16	2	7.83	-3.0
2	b2-microglobulin, human	MGHUB2	7	8	4	8	5	6.09	-5.0
3	Carbamoyl-phosphate synthase, rat	SYRTCA	72	96	28	95	56	5.97	-5.5
4	Proalbumin (serum albumin precursor), rat	ABRTS	32	57	15	53	27	5.98	-6.2
5	Serum albumin, rat	ABRTS	32	57	15	53	24	5.71	-9.0
6	Superoxid dismutase (Cu-Zn, SOD), rat	A26810	8	11	10	9	4	5.91	-9.2
7	Phospholipase C, phophoinosmoe-specific (?), rat	A28807	34	42	9	49	21	5.92	-9.2
8	Albumin, human	ABHUS	36	61	16	60	24	5.70	-11.9
9	Apo A-I lipoprotein, rat	A24700	18	24	6	23	12	5.32	-13.7
10	proApo A-I lipoprotein, human	LPHUA1	16	30	6	21	17	5.35	-14.3
11	NADPH cytochrome P-450 reductase, rat	RDRTO4	41	60	21	38	36	5.07	-15.6
12	Retinol binding protein, human	VAHU	18	10	2	10	14	5.04	-16.9
13	Actin beta, rat	ATRTC	23	26	9	19	18	5.06	-17.2
14	Actin gamma, rat	ATRTC	20	29	9	19	18	5.07	-16.8
15	Apo A-I lipoprotein, human	LPHUA1	16	30	5	21	16	5.10	-17.5
16	Apo A-IV lipoprotein, human	LPHUA4	20	49	8	28	24	4.88	-19.7
17	Tubulin alpha, rat	UBRTA	27	37	13	19	21	4.66	-19.8
18	F1ATPase beta, bovine	PWBOB	25	36	9	22	22	4.80	-21.0
19	Tubulin beta, pig	UBPGB	26	36	10	15	22	4.49	-22.5
20	Protein disulphide isomerase (PDI), rat hepatic	ISRTSS	43	51	11	51	9	4.07	-25.0
21	Cytochrome b5, rat	CBRT5	10	15	6	10	4	4.59	-26.0
22	Apo C-II lipoprotein, human	LPHUC2	4	7	0	6	1	4.44	-30.5
	Amino acid pI assumed in calulation:		3.9	4.1	6.0	10.8	12.5		

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An updated two-dimensional gel database of rat liver proteins useful in gene regulation and drug effect studies

We have improved upon the reference two-dimensional (2-D) electrophoretic map of rat liver proteins originally published in 1991 (N. L. Anderson *et al.*, *Electrophoresis* 1991, *12*, 907–930). A total of 53 proteins (102 spots) are now identified, many by microsequencing. In most cases, spots cut from wet, Coomassie Blue stained 2-D gels were submitted to internal tryptic digestion [2], and individual peptides, separated by high-performance liquid chromatography (HPLC), were sequenced using a Perkin-Elmer 477A sequenator. Additional spots were identified using specific antibodies.

Figure 1 shows the current annotated 2-D map of F344 rat liver, analyzed using the Iso-DALT system (20 × 25 cm gels) and BDH 4–8 carrier ampholytes. Both the map itself and the master spot number system remain the same as shown in the original publication. Table 1 lists the important features of each identification shown, including the gel position, p*I*, and M_r for the most abundant or most basic form of each protein. Using this extended base of identified spots, a series of four improved calibration functions has been derived for the p*I* and SDS-M_r axes (the first two of which are shown in Fig. 2A and B). Both forward and reverse functions are derived, so that one can compute the physical properties of a spot with a given gel location, or inversely compute the gel position expected for a protein having given physical properties:

$$Y_{\text{RATLIVER}} = f_{M_r\text{–RATLIVER Y}}(M_{r,\text{SEQUENCE-DERIVED}}) \quad (1)$$

$$X_{\text{RATLIVER}} = f_{p\text{I–RATLIVER X}}(pI_{\text{SEQUENCE-DERIVED}}) \quad (2)$$

$$M_{r,\text{GEL-DERIVED}} = f_{\text{RATLIVER Y–}M_r}(Y_{\text{RATLIVER}}) \quad (3)$$

$$pI_{\text{GEL-DERIVED}} = f_{\text{RATLIVER X–}pI}(X_{\text{RATLIVER}}) \quad (4)$$

A spreadsheet program (in Microsoft Excel) was developed to facilitate flexible computation of p*I*'s from amino acid sequence data, and the results were entered into a relational database (Microsoft Access). A table of spot positions and sequence-derived pI's and M_r's was fitted with a large series of analytic equations using Tablecurve (Jandel Scientific), and the four conversion Eqs. (1)–(4), relating computed p*I* and gel *X* coordinate, or computed molecular weight and gel *Y* coordinate, were selected, based on criteria of simplicity, goodness of fit and favorable asymptotic behavior. Table 2 lists the equations and coefficients. Application of Eqs. (3) and (4) to a spot's *X* and *Y* coordinates, given in [1], produce improved M_r estimates, and allow computation of p*I* directly in pH units, instead of in terms of positions relative to creatine phosphokinase (CPK) charge standards. The inverse Eqs. (1) and (2) were used to compute the gel positions of a series of p*I* and M_r tick marks. These tick marks were plotted with SigmaPlot (Jandel), together with fiducial marks locating several prominent spots, and the resulting graphic was aligned over the synthetic gel image (computed by Kepler from the master gel pattern) using Freelance (Lotus Development). Maps were printed as Postscript output from Freelance, either in black and white (as shown here) or in color, where label color indicates subcellular location (available from the first author upon request). We have also used the rat liver 2-D pattern as presented here to calibrate the patterns of other samples. Using mixtures of rat liver and mouse liver samples, for example, we made composite 2-D patterns that allow use of the rat pattern to standardize both axes of the mouse pattern. This was accomplished by deriving transformations relating the rat and mouse *X*, and separately the rat and mouse *Y*, axes (Table 2, lower half; Fig. 2C and D) based on a series of spots that coelectrophorese in these closely related species. These functions were then applied to derive equations relating the mouse liver *X* and *Y* to p*I* and SDS-M_r (Eqs. 5 and 6 below). The resulting standardized 2-D pattern for B6C3F1 mouse liver is shown in Fig. 3.

$$M_{r,\text{MOUSELIVER}} = f_{\text{RATLIVER Y–}M_r}(f_{\text{MOUSELIVER Y–RATLIVER Y}}(Y_{\text{MOUSELIVER}})) \quad (5)$$

$$pI_{\text{MOUSELIVER}} = f_{\text{RATLIVER X–}pI}(f_{\text{MOUSELIVER X–RATLIVER X}}(X_{\text{MOUSE LIVER}})) \quad (6)$$

A slightly more complex approach can be used to standardize samples that have few or no spots co-electrophoresing with rat liver proteins. In this case, a 2-D gel is prepared with a mixture of the two samples, and four functions (forward and backward, each for *X* and *Y*) are derived relating each sample's own master pattern to the composite. The required functions are then applied in a nested fashion to yield the desired result (using rat plasma as an example):

$$M_{r,\text{RATPLASMA}} = f_{\text{RATLIVER Y–}M_r}(f_{\text{RATPLASMA+LIVER Y–RATLIVER Y}}(f_{\text{RATPLASMA Y–RATPLASMA+LIVER Y}}(Y_{\text{RATPLASMA}}))) \quad (7)$$

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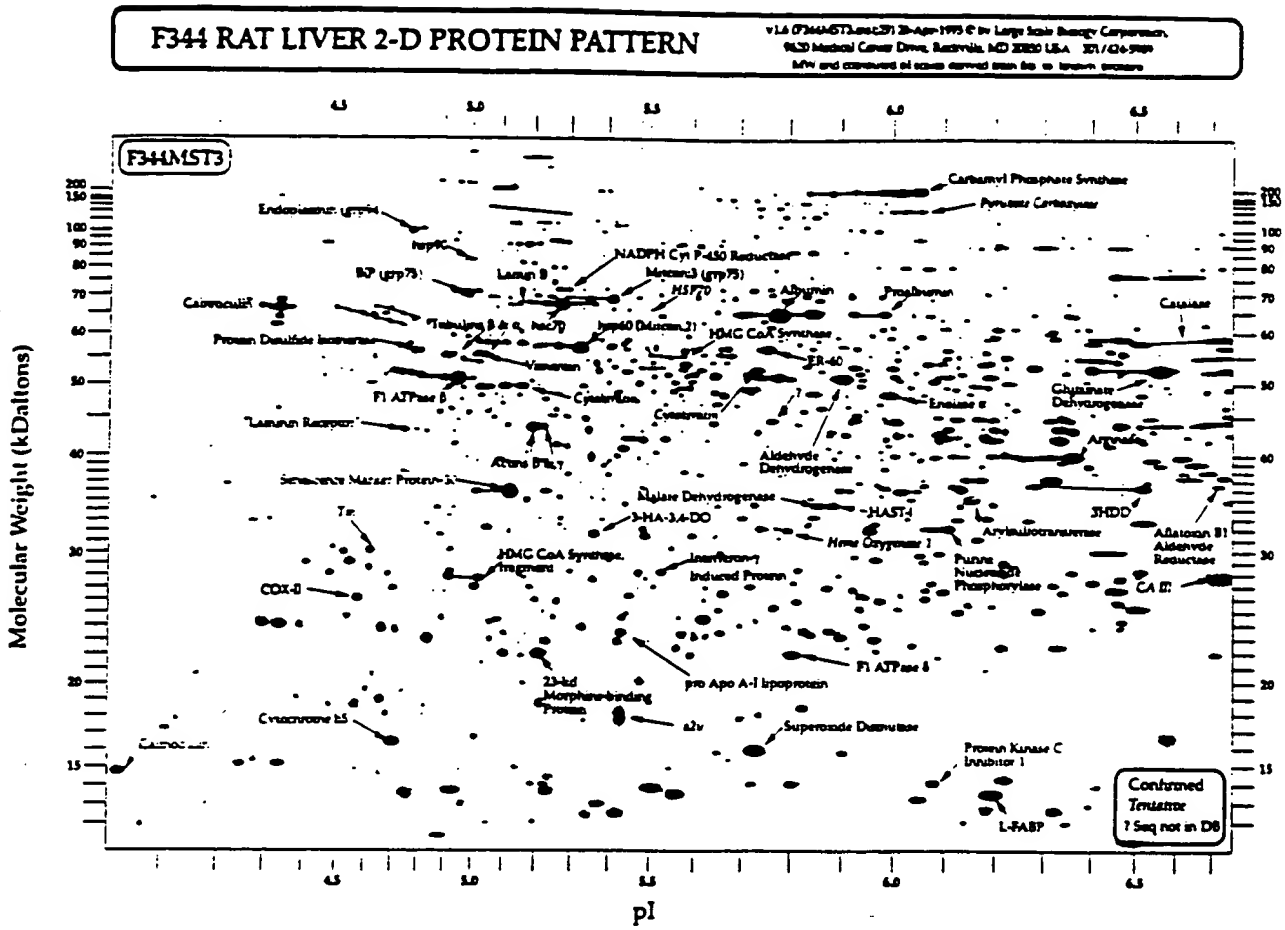

Figure 1. Master 2-D gel pattern of Fischer 344 rat liver proteins, annotated with 53 protein identifications and computed p*I* and M_r axes. Tentative identifications are in italic type.

Table 1. Proteins identified in the 2-D pattern of F344 rat liver

MSN[a]	Protein ID[b]	Protein name	Identification comments	Gel X[c]	Experimental p*I*[d]	Gel Y[c]	Experimental M_r[d]
126	HADO-HUMAN[e]	3-HA-3,4-DO: 3-hydroxyanthranilate-3,4-dioxygenase	Internal sequence	871.95	5.36	921.35	30 207
137, 159, 288, 258	DIDH_RAT	3HDD: 3-hydroxysteroid dihydrodiol reductase	Ab (T.M. Penning) and pure protein	1857.52	6.51	822.52	34 406
173	MUP_RAT	a_{2u} globulin	Presence in liver microsome lumen, abundance in kidney, p*I*, M_r	919.16	5.43	1313.81	19 549
38	ACTB_HUMAN	Actin β	Analogy with other mammalian patterns (*e.g.* human) through coelectrophoresis	763.40	5.19	693.64	41 586
68	ACTG_HUMAN	Actin γ	Analogy with other mammalian patterns (*e.g.* human) through coelectrophoresis	779.42	5.21	692.26	41 677
693	AFAR_RAT	Aflatoxin B1 aldehyde reductase	Internal sequence	1993.32	6.72	818.60	34 593
28, 21, 33	ALBU_RAT	Albumin	Coelectrophoresis with principal plasma protein	1262.81	5.86	445.64	66 354
43	DHAM_RAT	Aldehyde dehydrogenase	*N*-Terminal sequence and AAA	1317.72	5.91	589.03	49 602
96	ARGI_RAT	Arginase	Internal sequence	1730.72	6.34	756.02	37 819
117	SUAR_RAT	Arylsulfotransferase	Internal sequence	1547.96	6.14	849.08	33 186
1163, 1161, 1162, 20	GR78_RAT	BIP (GRP-78)	Ab (F. Witzmann)	665.33	5.01	397.39	74 564
185	CAH3_RAT	CA-III	Uncertain; by comparison with mouse	1996.60	6.72	1017.02	26 887
123	CALM_HUMAN	Calmodulin	Analogy with human cellular patterns through coelectrophoresis	23.05	4.03	1433.25	17 419
3, 201, 48, 39, 22, 24	CRTC_RAT	Calreticulin	Ab (Lance Pohl)	310.59	4.34	433.80	68 206

Table 1. continued

MSN[a]	Protein IDb)	Protein name	Identification comments	Gel *X*[c]	Experimental p*I*[d]	Gel *Y*[c]	Experimental M_r[d]
1184, 1186, 114, 174, 118 5, 167, 157	CPSM_RAT	Carbamyl phosphate synthase	2-D of pure protein; comfirmed by *N*-terminal sequence and AAA	1453.56	6.05	181.64	160 640
54, 61	CATA_RAT	Catalase	Internal sequence	2000.81	6.73	499.64	58 968
136	COX2_RAT	COX-II	Ab (J. W. Taanman), confirmed by internal sequence	452.57	4.61	1062.67	25 504
87	CYB5_RAT	Cytochrome B5	2-D of pure protein; Ab; confirmed by AAA	515.68	4.73	1370.55	18 493
41	CK-RAT[e]	Cytokeratin	Location in cytoskeletal fraction	1165.12	5.75	569.09	51 448
29	CK-RAT[e]	Cytokeratin	Location in cytoskeletal fraction	743.11	5.15	605.23	48 187
5, 11	ENPL-RAT[e]	Endoplasmin	Ab (F. Witzmann)	567.73	4.83	263.37	112 194
60	ENOA_RAT	Enolase A	Internal sequence and AAA	1399.78	6.00	623.54	46 674
27	ER60_RAT	ER-60	*N*-Terminal sequence (R. M. Van Frank)	1184.20	5.77	523.51	56.169
17	ATPB_RAT	F1 ATPase β	*N*-Terminal sequence and AAA	629.06	4.95	588.83	49 620
196	ATP7_RAT	F1 ATPase δ	Internal sequence	1227.24	5.82	1184.65	22 310
79	F16P_RAT	Fructose-1.6-bis-phosphatase	Uncertain; by comparison with ID in Garrison and Wager (JBC 257:13135-13143)	924.54	5.44	737.77	38 858
62, 78	DHE3_RAT	Glutamate dehydrogenase	*N*-Terminal sequence and internal sequence	1887.39	6.55	566.92	51 655
125	HAST-RAT[e]	HAST-1: N-hydroxyaryl-amine sulfotransferase	Internal sequence	1297.94	5.89	861.55	32 638
307	HO1_RAT	Heme oxygenase 1	Uncertain; available data from internal sequence	1219.39	5.81	915.71	30 423
413, 1250, 933	HMCS_RAT	HMG CoA synthase, cytosolic	Ab (J. Germershausen)	1033.48	5.59	538.13	54 571
133, 144, 235	HMCS_RAT	HMG CoA synthase, mitochondrial (frag)	Ab (J. Germershausen), *N*-terminal sequence (Steiner/Lottspeich)	666.40	5.02	1019.42	26 811
8, 23, 1307	HS7C_RAT	HSC-70	Positional homology (with human, *etc.*) through coelectrophoresis	811.87	5.27	425.76	69 521
15, 25, 110	P60_RAT	HSP-60	Ab (F. Witzman); confirmed by *N*-terminal sequence and AAA	845.09	5.32	520.03	56 561
971	HS70-RAT[e]	HSP-70	Ab (F. Witzman)	976.11	5.51	437.14	67 674
1216, 1215, 90	HS90-RAT[e]	HSP-90	Ab (F. Witzman)	659.86	5.00	329	90 107
256	INGI-HUMAN	Interferon-γ induced protein	Internal sequence	993.85	5.54	1006.04	27 237
415, 734	LAMB-RAT[e]	Lamin B	Positional homology with human through coelectrophoresis, nuclear location	737.10	5.14	425.19	69 615
80	LAMR-RAT[e]	"Laminin receptor"	Internal sequence	534.02	4.77	697.62	41 327
227	FABL_RAT	L-FABP (liver fatty acid binding protein)	Ab (N. M. Bass)	1586.09	6.18	1483.43	16 622
134	MDHC_MOUSE	Malate dehydrogenase	Internal sequence	1270.85	5.86	861.96	32 620
18, 35, 226	GR75-RAT[e]	Mitcon:3; grp75	Positional homology with human through coelectrophoresis	905.67	5.41	413.67	71 589
175, 251	NCPR_RAT	NADPH P450 reductase	2-D of pure protein	824.69	5.29	393.21	75 366
1168, 1170, 1171	PDI_RAT	PDI: Protein disulfide isomerase	*N*-Terminal sequence (R. M. van Frank), Ab	564.30	4.83	528.47	55 618
47, 93	ALBU_RAT	Pro-Albumin	Microsomal lumen location, p*I*, M_r relative to albumin	1391.03	5.99	446.68	66 195
236	APA1_RAT	Pro-APO A-I lipoprotein	Coelectrophoresis with plasma protein	920.41	5.43	1137.51	23 467
320	IPK1_BOVIN	Protein kinase C inhibitor 1	Internal sequence; homology with bovine protein	1480.01	6.08	1458.81	17 007
152	PNPH_MOUSE	Purine nucleoside phosphorylase	Internal sequence	1507.19	6.10	911.16	30 599
1179, 1180, 1181, 1182, 1183	PYVC-RAT[e]	Pyruvate carboxylase	Tentative; 2-D of pure protein (J. G. Henslee, *JBC*, 1979); reported in *Biochim. Biophys. Acta 1022*, 115–125.	1485.10	6.08	223.52	131 589
55, 103	SM30_RAT	SMP-30: Senescence marker protein-30	Internal sequence	721.71	5.11	830.10	34 051
135	SODC_RAT	Superoxide dismutase	AAA; comfirmed by internal sequence (R. M. Van Frank)	1161.24	5.74	1388.68	18 173
172	TPM-RAT[e]	Tm: tropomyosin	Location in cytoskeleton, 2-D position relative to human, Ab	476.24	4.66	957.86	28 865
277, 56	TBA1_RAT	Tubulin α	Positional homology with human through coelectrophoresis, cytoskeletal location	688.22	5.06	537.67	54 620
50, 1225	TBB1_RAT	Tubulin β	Positional homology with human through coelectrophoresis, cytoskeletal location	621.29	4.93	535.48	54 855
1224	VIME_RAT	Vimentin	Positonal homology with human through coelectrophoresis, cytoskeletal location	673.00	5.03	539.50	54 426

Table 1. continued

MSN[a]	Protein ID[b]	Protein name	Identification comments	Gel X[c]	Experimental pI[d]	Gel Y[c]	Experimental M_r[d]
113	Unknown	?: not in sequence databases	Internal sequence	1191.28	5.78	680.42	42 469
104	BBPL_RAT	23 kDa morphine-binding protein	Internal sequence	773.31	5.20	1182.41	22 363

a) Master spot number (MSN) from [1]
b) SwissPROT identifier
c) Coordinates of the most basic or most abundant assigned spot on the F344 master gel pattern
d) pI and M_r of the most basic or most abundant assigned spot, derived from the calibration functions included here
e) SwissPROT style proposed identifier
Abbreviations: AAA, amino acid analysis; Ab, antibody

Table 2. Equations and coefficients

Function	Equation (f)	r2	a	b	c	d	e
Rat gel Y = f(computed M_r)	$y = a + b\exp(-x/c)$	0.988181021	178.74803	1967.7892	32363.958		
Rat gel X = f(computed pI)	$y = a + bx + cx/\ln x + d/x + e/x^{1.5}$	0.99247216	−8685665.5	−904497.94	3856926.1	18276844	−27154534
Computed M_r = f(rat gel Y)	$y = a + bxc$	0.9960177	−8464.5809	19095881	−0.9086255		
Computed pI = f(rat gel X)	$y = a + bx + cx^2 + dx^2 \ln x + ex^3$	0.99176499	4.044686	−0.00114238	0.0000323	−0.00000455	0.00000000176
Mouse gel Y = f(rat gel Y)	$y = a + bx + cx^{2.5} + dx^{0.5} \ln x + cx/\ln x$	0.99951069	11861.44	678.91666	−0.78964914	1567.5639	−6953.9592
Mouse gel X = f(rat gel X)	$y = a + bx^2 \ln x + cx^{2.5} + dx^3$	0.99926349	58.935923	0.00091353	−0.000213688	0.00000159	
Rat gel Y = f(mouse gel Y)	$y = a + bx^2 \ln x + cx^{2.5} + dx^3$	0.99950032	69.740526	0.00050772	−0.000130392	0.00000116	
Rat gel X = f(mouse gel X)	$y = a + bx + cx^2 \ln x + dx^{2.5} + ex^3$	0.9992832	−198.07189	2.0899063	−0.000671191	0.000145189	−0.000000986

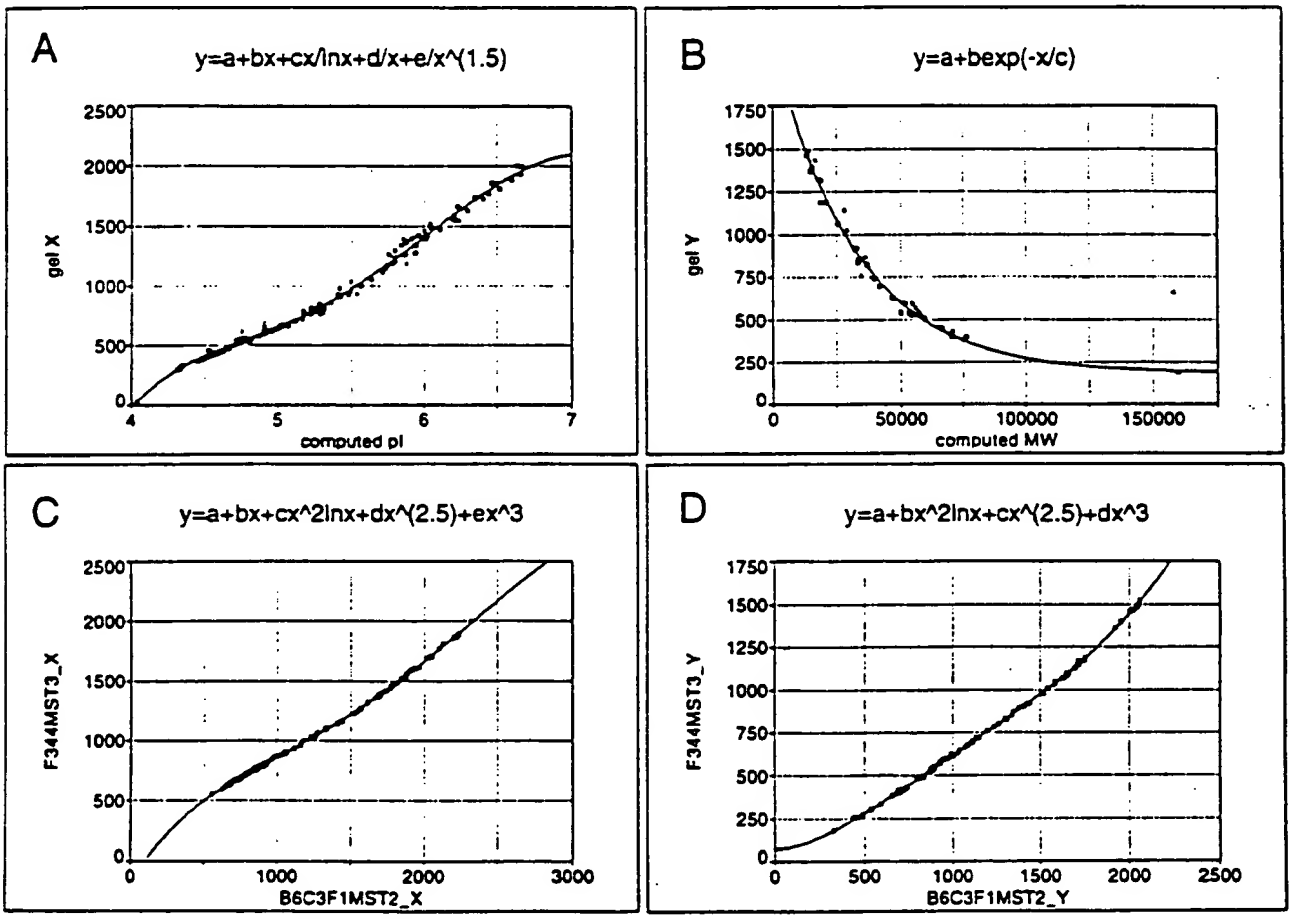

Figure 2. Plots showing fits of selected equations (continuous curves) to data on identified proteins (square symbols). (A) pI computed from sequence data *versus* gel *X* position for identified spots in F344 rat liver; (B) M_r computed from sequence data *versus* gel *Y* position for identified spots in F344 rat liver; (C) gel *X* position for spots in B6C3F1 mouse liver *versus X* position in F3443 rat liver, for coelectrophoresing spots; (D) gel *Y* position for spots in B6C3F1 mouse liver *versus Y* position in F3443 rat liver, for coelectrophoresing spots. In each case, inverse equations were also computed (Table 2).

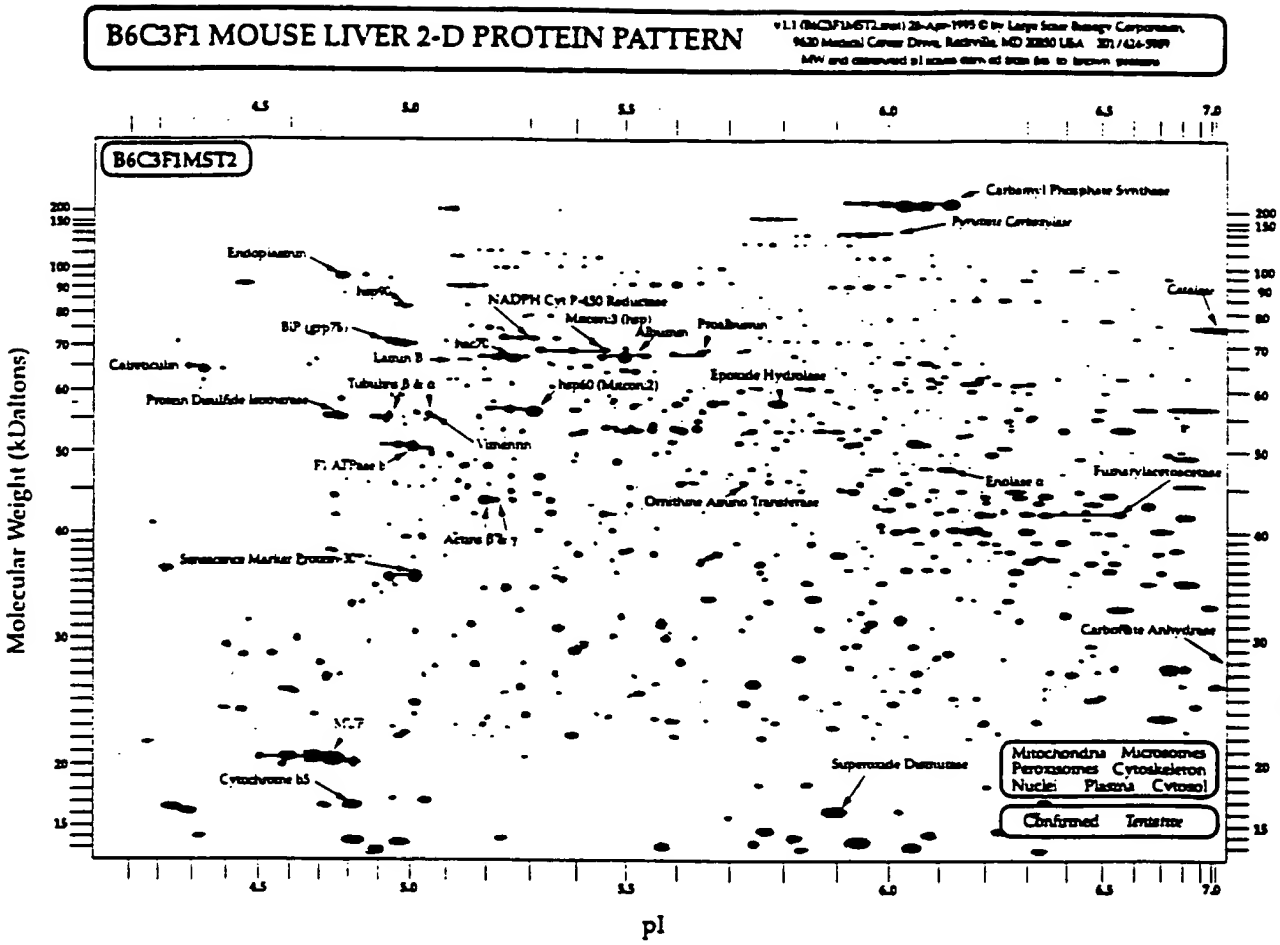

Figure 3. Master 2-D gel pattern for B6C3F1 mouse liver, standardized using the F344 rat liver pattern identifications, according to the method described in the text. Twenty-nine proteins are identified.

$$pI_{RAT\,PLASMA} = f_{RAT\,LIVER\ X-pI}(f_{RAT\,PLASMA-LIVER\ X-RAT\,LIVER\ X}(f_{RAT\,PLASMA\ X-RAT\,PLASMA-LIVER\ X}(X_{RAT\,PLASMA}))) \quad (8)$$

This unified approach, in which one well-populated 2-D pattern is used to standardize a family of other patterns, has the additional advantage that the resulting p*I* and M_r scales are directly compatible. Hence one can compare the relative p*I*'s of mouse and rat versions of a sequenced protein in a consistent p*I* measurement system, and select likely inter-species analogs based on positional relationships on common scales. Adoption of immobilized pH gradient (IPG) technology [4–7] will result in substantial improvements in p*I* positional reproducibility for standard 2-D maps such as those presented here; however, we believe that our approach will continue to be useful in establishing the empirical pH gradient actually achieved by such gels under given experimental conditions (temperature, urea concentration, *etc.*), in relating patterns run on different IPG ranges and using different lots of IPG gels (between which some variation will persist). Development of rodent organ maps is a continuing effort in our laboratories [8–10], and results in regular additions of identified proteins. Those who wish to receive current rodent liver maps, with color annotations, should send a stamped self-addressed envelope to the first author.

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Company Info

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Large Scale Biology Corporation is the leader in the integrated discovery, production and application of proteins - the functional units of all biological processes.

Large Scale Biology Corporation (LSB, Vacaville, CA) and its subsidiary Large Scale Proteomics Corp. (LSP, Germantown, MD) are a biotechnology enterprise with the mission of accelerating the speed and productivity of the life sciences industry product discovery and development programs. Unique among biotechnology companies is LSB's integration of technologies to discover, analyze, manufacture and find new applications for proteins - the functional units of all biological processes.

Genomics companies have focused on deciphering genetic information, providing an initial but only partial understanding of biological processes. LSB's proprietary protein technologies can enable the transformation of genomic information into products such as drug targets, therapeutics, diagnostics for drug efficacy and toxicity, and traits for agricultural crops. Large Scale Biology has gone beyond the "genomics" realm in its business model and developed ways to integrate the discovery of gene function with quantitative protein analysis and protein manufacturing. This integration of technology platforms favorably positions LSB as a leading provider of valuable content to industry leaders in the fields of diagnostics, therapeutics, vaccines and agribusiness.

LSB was founded in 1987 with the goal of commercializing its proprietary GENEWARE viral vector system - a novel technology for gene expression. Using safe RNA viruses to transiently express genes in non-recombinant plants, LSB has positioned itself in the industry to provide cost-effective manufacturing and purification of diverse protein and peptide products. The same technology can be applied to the expression of libraries of foreign genes in an automated, high-throughput format to discover the function of genes with unparalleled efficiency. The GENEWARE system and associated proprietary technologies form the basis for LSB's functional genomics, biomanufacturing and a variety of proprietary products under development.

From its foundation, LSB understood the need to integrate functional genomic and protein manufacturing expertise with quantitative protein analysis and informatics to become a world-leader in the protein field. In 1999, LSB acquired a privately held pharmaceutical proteomics company originally founded in 1985. Large Scale Proteomics Corporation (a wholly

owned subsidiary of Large Scale Biology Corporation) is an industry leader in identifying and characterizing proteins in all types of biological samples for the discovery and development of new and more effective therapies, diagnostics, and agricultural products.

"Proteomics" is the study of the entire complement of proteins expressed in a cell, tissue, or organism. Proteomics can significantly improve drug discovery and development because most illness is associated with imbalances among, or malfunctions of, proteins. Only a small fraction of diseases can be attributed to the presence of a defective gene. Unlike classical genomics approaches that discover genes that may relate to a disease, LSP has developed a proprietary system called the ProGEx module for directly characterizing proteins associated with disease. Using this same technology, LSP can characterize the effects of candidate drugs intended to reverse a disease process, and to determine the degree to which this objective is achieved free of adverse side effects.

LSB and LSP have protected their many discoveries though an extensive portfolio of domestic and foreign patents and have developed commercial alliances and partnerships to exploit the value of their technologies. LSB and LSP scientists and engineers focus on the development and application of resources to help clients meet their objectives as well as the development of our own proprietary products for subsequent partnering with industry leaders.

A combined staff of 140 professionals operates from three locations in the United States, with a network of collaborators and affiliates throughout the US and Europe. Company headquarters, R&D laboratories and its Genomics division are located in Vacaville, California about 60 miles northeast of San Francisco. Process development and biomanufacturing take place in Owensboro, Kentucky, and LSB's Large Scale Proteomics Corporation subsidiary is located in Germantown, Maryland.

In August, 2000, LSB completed an initial public offering (IPO) of 5 million shares of common stock and now trades on the NASDAQ under the symbol LSBC.

Leadership - Large Scale Biology Corporation

Robert L. Erwin, Chairman of the Board and Chief Executive Officer, founded LSB™ and has served as a director and officer since 1987. Mr. Erwin is the former chairman of the State of California Breast Cancer Research Council and currently serves on the University of California President's Engineering Advisory Council. He is Chairman of the Supervisory Board of Icon Genetics AG. As a co-founder of Sungene Technologies Corp., Mr. Erwin served as Vice President of Research and Product Development from 1981 through 1986. He has served on the Biotechnology Industry Advisory Board for Iowa State University. Mr. Erwin received his M.S. degree in Genetics from Louisiana State University and is an inventor on several LSB patents.

*David R. McGee, Ph.D.,*a co-founder of LSB and Senior Vice President and Chief Operating Officer, has been an officer since 1987. Prior to joining LSB, Dr. McGee was Vice President of Operations at Sungene Technologies Corporation from 1983 to 1987. Dr. McGee received his Ph.D. in Genetics from Louisiana State University and served as a faculty instructor of zoology and genetics at Louisiana State University.

*Laurence K. Grill, Ph.D.,*a co-founder of LSB and Senior Vice President, Research and Development, has served as an officer since 1987. Dr. Grill was the Manager of Plant Molecular Biology for Sandoz Crop Protection Corp. from 1984 to 1987 and Senior Research

Scientist in the Department of Molecular Biology at Zoecon Research Institute from 1980 to 1984. He received his Ph.D. from the University of California at Riverside with an emphasis on the molecular basis for viral gene expression in plants.

R. Barry Holtz, Ph. D., Senior Vice President, Biopharmaceutical Manufacturing, has served the company as an officer since 1989 upon the acquisition of Holtz Bio-Engineering, which was founded in 1980. Dr. Holtz was a co-founder and Director of Research for MFI, Inc., the largest manufacturer of microencapsulated nutrients for agriculture and Director of Fundamental Research at Foremost-McKesson, Inc. Dr. Holtz received his Ph.D. in Biochemistry from Pennsylvania State University and served as Assistant Professor in the Department of Food Science and Nutrition at Ohio State University.

Daniel Tusé, Ph.D., has been an officer of LSB since he joined the Company in 1995 as Vice President, Pharmaceutical Development. Dr. Tusé manages the company's pharmaceutical design and development programs, including LSB's novel vaccines and immunotherapeutics initiatives. Prior to joining LSB, Dr. Tusé was Assistant Director of SRI International's (Menlo Park, Calif.) Life Sciences Division. In his 17 years at SRI, Dr. Tusé developed extensive R&D experience in pharmaceuticals and specialty chemicals, serving an international list of clients. Dr. Tusé received his Ph.D. in Microbiology (1980, *cum laude*) with a minor in Toxicology from the University of California, Davis.

John S. Rakitan, a co-founder of LSB, Senior Vice President & General Counsel and Secretary, has served as an officer since 1988. Prior to joining LSB, Mr. Rakitan was an attorney in private practice. Mr. Rakitan received his J.D. degree from the University of Notre Dame.

Michael D. Centron, Treasurer, has served as Controller since 1988 and was elected as Treasurer in 1991. Mr. Centron was Audit Supervisor for Varian Associates from June 1985 through July 1988, and he also worked for Arthur Young and Co. (currently Ernst & Young). Mr. Centron is a certified public accountant and received his M.B.A. degree from the University of California at Berkeley.

Guy della-Cioppa, Ph.D., is an officer of the company and currently serves as Vice President, Genomics. Prior to joining the company in 1989, Dr. della-Cioppa worked for Monsanto Company in St. Louis, MO from 1984-1989 and was an NIH Postdoctoral Fellow at the Worcester Foundation for Experimental Biology in Shrewsbury, MA from 1983-1984. He received his Ph.D. in Biology from the University of California, Los Angeles.

William M. Pfann joined Large Scale Biology in August 2000 as Senior Vice President Finance and Chief Financial Officer. Mr. Pfann was formerly with PricewaterhouseCoopers LLP from 1969 to July 2000, most recently as the Risk Management Partner for the Western Region. He served in a number of management roles at PwC, including leader of the firm's Silicon Valley audit practice, National Director of the networking and communications sector and Managing Partner of the Northern California emerging business group, as well as Partner-in-Charge of the Oakland and Walnut Creek, California offices. Mr. Pfann received a B.S. degree from the University of California, Berkeley, in Business Administration and an MBA in Accounting from Golden Gate University.

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Large Scale Proteomics Corporation

Leadership - Large Scale Proteomics Corporation

*N. Leigh Anderson, Ph D.,*Chairman, President and CEO of Large Scale Proteomics Corporation (LSP™). Dr. Anderson obtained his B.A. in Physics with honors from Yale and a Ph.D. in Molecular Biology from Cambridge University (England) working with M. F. Perutz as a Churchill Fellow at the MRC Laboratory of Molecular Biology. Subsequently he co-founded the Molecular Anatomy Program at the Argonne National Laboratory (Chicago) where his work in the development of 2-dimensional electrophoresis (2-DE) and molecular database technology earned him, among other distinctions, the American Association for Clinical Chemistry's Young Investigator Award for 1982 and the 1983 Pittsburgh Analytical Chemistry Award. In 1985 Dr. Anderson co-founded LSP (originally Large Scale Biology Corp., Germantown, MD) in order to pursue commercial development and large-scale applications of 2-D electrophoretic protein mapping technology.

*Norman G. Anderson, Ph.D.,*Chief Scientist at LSP. Dr. Anderson has a distinguished record as an inventor. His career includes senior positions at Oak Ridge and Argonne National Laboratories (ORNL and ANL), more than 300 scientific publications, and the receipt of more than 20 prestigious awards in recognition of his work in science and technology. For his invention of the zonal ultracentrifuge, he received the John Scott Medal Award, and for the centrifugal fast analyzer, the Preis Biochemische Analytik für Klinische Chemie from Die Deutsche Gesellschaft für Klinische Chemie for the most outstanding analytical development in clinical chemistry worldwide during a 2-year period. In 1984 ANL awarded him its career patent leader award for the largest number of patents issued to an employee. At that time the commercial value of his inventions in terms of U.S. sales and royalties from foreign licensing were $250 million and $1 million, respectively. Dr. Anderson received his degrees at Duke University: a B.A. in Zoology, M.A. in Physiology, and Ph.D. in Cell Physiology. He holds 28 patents.

*Constance Seniff,*Vice President, Operations. Ms. Seniff has managed LSP's operations since 1993. Her background includes thirteen years in international business prior to joining LSP, five abroad in the employ of foreign firms. Ms. Seniff is responsible for helping formulate and implement business development and database commercialization strategies for LSP in coordination with the management of LSP's parent company, Large Scale Biology Corporation. Ms. Seniff has a B.Sc. degree in Business (with honors) from Florida State University.

Robert J. Walden, Vice President, Finance at LSP. Mr. Walden joined LSP in 1997 and has served as a director since 1999. He previously served as Vice President of Finance and Administration at Osiris Therapeutics, Inc., and as Chief Financial Officer at the American Type Culture Collection (ATCC). Mr. Walden received his degree in Finance from the University of Maryland.

*Jean-Paul Hofmann, Ph.D.,*Vice President, Software Development at LSP. Dr. Hofmann is a plant geneticist by training, having earned a B.S. in Biology, M.S. in Biochemistry and Genetics, and Ph.D. in Plant Genetics from the University of Orsay, Paris. He has extensive

experience in using 2-DE in agronomic research and in designing analytical software for 1- and 2-D applications. He has held senior scientific positions in industry and research institutes, in the U.S., France and the Ivory Coast.

John Taylor, Ph.D., Vice President, Software Development and Bioinformatics. Dr. Taylor is the principal developer of Kepler™, LSP's analytical software for automated 2-DE pattern analysis. Prior to joining LSB, Dr. Taylor served as computer scientist in the Molecular Anatomy Program at Argonne, and on the research staffs of the University of Chicago and the Armed Forces Institute of Pathology in Washington, D.C. Dr. Taylor received a B.S. in Physics from the University of South Carolina, and a Ph.D. in Nuclear Physics from Duke University.

Sandra Steiner, Ph.D., currently serves as Vice President Proteomics Applications. Prior to joining the Company, Dr. Steiner founded and directed the Molecular Toxicology Group at Novartis in Basel, Switzerland and was a member in several multi-disciplinary drug development project teams. Dr. Steiner received her Ph.D. in Toxicology/Pharmacology from the University of Basel, Switzerland.

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Human cellular protein patterns and their link to genome DNA sequence data: usefulness of two-dimensional gel electrophoresis and microsequencing

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ABSTRACT **Analysis of cellular protein patterns by computer-aided 2-dimensional gel electrophoresis together with recent advances in protein sequence analysis have made possible the establishment of comprehensive 2-dimensional gel protein databases that may link protein and DNA information and that offer a global approach to the study of the cell. Using the integrated approach offered by 2-dimensional gel protein databases it is now possible to reveal phenotype specific protein (or proteins), to microsequence them, to search for homology with previously identified proteins, to clone the cDNAs, to assign partial protein sequence to genes for which the full DNA sequence and the chromosome location is known, and to study the regulatory properties and function of groups of proteins that are coordinately expressed in a given biological process. Human 2-dimensional gel protein databases are becoming increasingly important in view of the concerted effort to map and sequence the entire genome.—Celis, J. E.; Rasmussen, H. H.; Leffers, H.; Madsen, P.; Honoré, B.; Gesser, B.; Dejgaard, K.; Vandekerckhove, J. Human cellular protein patterns and their link to genome DNA sequence data: usefulness of two-dimensional gel electrophoresis and microsequencing. *FASEB J.* 5: 2200-2208; 1991.**

Key Words: human protein patterns · 2-dimensional gel protein databases · gene expression · microsequencing · cDNA cloning · linking protein and DNA information · genome mapping and sequencing

PROTEINS SYNTHESIZED FROM information contained in the DNA orchestrate most cellular functions. The total number of proteins synthesized by a typical human cell is unknown although current estimates range from 3000 to 6000. Of these, as many as 70% may perform household functions and are expected to be shared by all cell types irrespective of their origin. There are many different cell types in the human body with perhaps 30,000 to 50,000 proteins expressed in the organism as a whole judged from the fact that about 3% of the haploid genome correspond to genes. Today only a small fraction of the total set of proteins has been identified, and little is known about the protein patterns of individual cell types or their variation under physiological and abnormal conditions.

For the past 15 years, high resolution 2-dimensional gel electrophoresis has been the technique of choice to determine the protein composition of a given cell type and for monitoring changes in gene activity through quantitative and qualitative analysis of the thousands of proteins that orchestrate various cellular functions (refs 1-6 and references therein). The technique originally described by O'Farrell (.. separates proteins in terms of their isoelectric point (pI) an.. molecular weight. Usually one chooses a condition of interest and the cell reveals the global protein behavioral response as all detected proteins can be analyzed both qualitatively and quantitatively in relation to each other. At present, most available 2-dimensional gel techniques (regular gel format) can resolve between 1000 and 2000 proteins from a given mammalian cell type, a number that corresponds to about 2 million base pairs of coded DNA. Less abundant proteins can be detected by analyzing partiall purified cellular fractions.

Two-dimensional gel ectrophoresis has been widely applied to analysis of cellular protein patterns from bacteria to mammalian cells (refs 1-6, and references therein). In spite of much work, however, information gathered from these studies has not reached the scientific community in its fullness because of lack of standardized gel systems and the lack of means for storing and communicating protein information. Only recently, because of the development of appropriate computer software (7-13), has it been possible to scan gels, assign numbers to individual proteins, and store the wealth of information in quantitative and qualitative comprehensive 2-dimensional gel protein databases (4, 14-23), i.e., those containing information about the various properties (physical, chemical, biological, biochemical, physiological, genetic, immunological, architectural, etc.) of all the proteins that can be detected in a given cell type. Such integrated 2-dimensional gel protein databases offer an easy and standardized medium in which to store and communicate protein information and provide a unique framework in which to focus a multidisciplinary approach to study the cell. Once a protein is identified in the database, all of the information accumulated can be easily retrieved and made available to the researcher. In the long run, protein databases are expected to foster a wide variety of biological information that may be instrumental to researchers working in many areas of biology—among others, cancer and oncogene studies, differentiation, development, drug development and testing, genetic variation, and diagnosis of genetic and clinical diseases (**Fig. 1**).

The approach using systematic 2-dimensional gel protein analysis has recently gained a new dimension with the advent of techniques to microsequence major proteins recorded

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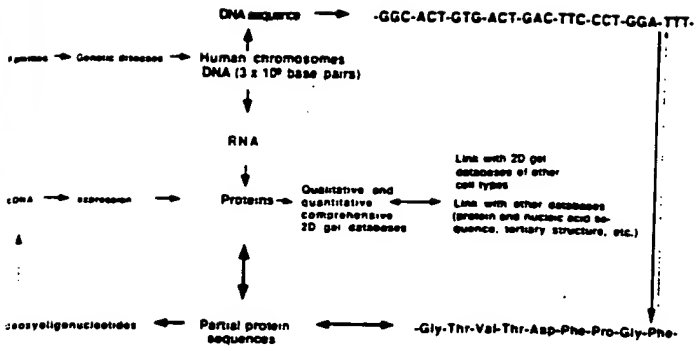

Figure 1. Interface between partial protein sequence databases, comprehensive 2-dimensional gel databases, and the human genome sequencing project. Appropriate software is required to compare protein and DNA sequences. In general, although the inference of a protein's sequence from the DNA sequence (thick arrow) is direct and unambiguous, the DNA sequence can only be inferred approximately from the protein sequence (thin arrow) and cloning of the gene requires either a cDNA or the requisite group of oligonucleotide probes deduced from the partial amino acid sequence. Modified from ref 6.

in the databases (refs 24–42 and references therein). Partial protein sequences can be used to search for protein identity as well as to prepare specific DNA probes for cloning as-yet-uncharacterized proteins (Fig. 1). As these sequences can be stored in the database (see for example Fig. 2H), they offer a unique opportunity to link information on proteins with the existing or forthcoming DNA sequence data on the human genome (Fig. 1) (20, 36, 39).

Using the integrated approach offered by comprehensive 2-dimensional gel databases (Fig. 1), it will be possible to identify phenotype-specific proteins; microsequence them and store the information in the database; search for homology with previously characterized proteins; clone the cDNAs, assign partial protein sequences to genes for which the full DNA sequence and the chromosome location are known, and study the regulatory properties and function of groups of proteins (pathways, organelles, etc.) that are coordinately expressed in a given biological process. Comprehensive 2-dimensional gel protein databases will depict an integrated picture of the expression levels and properties of the thousands of protein components of organelles, pathways, and cytoskeletal systems in both physiological and abnormal conditions and are expected to lead to identification of new regulatory networks in different cell types and organisms. In the future, 2-dimensional gel protein databases may be linked to each other as well as to national and international specialized databanks on nucleic acid and protein sequences, protein structures, NMR experimental data, complex carbohydrates, etc.

A few 2-dimensional gel protein databases that are accessible in a computer form have been published in extenso: these correspond to the protein-gene database of *Escherichia coli* K-12 developed by Neidhardt and colleagues (14, 23), the rat REF 52 database established by Garrels and co-workers at Cold Spring Harbor (18, 22), and a few human databases (transformed amnion cells [15, 20], normal embryonal lung MRC-5 fibroblasts [17, 21], keratinocytes [19] and peripheral blood mononuclear cells [15]) developed in Aarhus. Given space limitations and to keep this review in focus, we will concentrate on the computerized analysis of human cellular 2-dimensional gel patterns, and in particular on the steps involved in establishing comprehensive 2-dimensional gel databases that can link protein and DNA information.

MAKING AND MANAGING A COMPREHENSIVE 2-DIMENSIONAL GEL DATABASE OF HUMAN CELLULAR PROTEINS

The first step in making a comprehensive 2-dimensional gel protein database is to prepare a synthetic image (digital form of the gel image) of the gel (fluorogram, Coomassie blue or silver stained gel) to be used as a standard or master reference. This can be done with laser scanners, charge couple device (CCD)[2] array scanners, television cameras, rotating drum scanners, and multiwire chambers (13). Computerized analysis systems for spot detection, quantitation, pattern matching, and data handling (access and retrieval of information, database making) have been described in the literature (ELSIE [43], GELLAB [11], HERMeS [44], MELANIE [10], QUEST (9), and TYCHO [8]) and some are available commercially (PDQUEST, Protein Database Inc., Huntington, N.Y.; KEPLER, Large Scale Biology, Rockville, Md.; Visage, BioImage Corporation, Ann Arbor, Mich.; Gemini, Joyce Loebl, Gateshead; Microscan 1000, Technology Resources Inc., Nashville, Tenn. and MasterScan, Billerica, Mass.). Unfortunately, most of these systems are incompatible with one another and their advantages and disadvantages have been discussed by Miller (13).

In our work station in Aarhus, fluorograms are scanned with a Molecular Dynamics laser scanner and the data are analyzed using the PDQUEST II software (Protein Databases Inc.) (12) running on a spark station computer 4100 FC-8-P3 from SUN Microsystems, Inc. The scanner measures intensity in the range of 0–2.0 absorbance. A typical scan of a 17 × 17 cm fluorogram takes about 2 min. Steps in image analysis include: initial smoothing, background substraction, final smoothing, spot detection, and fitting of ideal Gaussian distribution to spot centers. Spot intensity is calculated as the integration of a fitted Gaussian. If calibration strips containing individual segments of a known amount of radioactivity are used, it is possible to merge multiple exposures of the sample image into a single data image of greater dynamic range. Once the synthetic image is created it can be stored on disk and displayed directly on the monitor. Functions that can be used to edit the images include: cancel (for example, to erase scratches that may have been interpreted as spots by the computer; cancel streaks or low dpm spots), combine (sometimes a spot may be resolved into several closely packed spots), restore, uncombine, and add spot to the gel. The process is time consuming—about 1-1/2 day per image. Edited standard images can be matched to other synthetic images. **Figure 2*A*** shows a portion of a standard synthetic image (IEF) of a fluorogram of [^{35}S]methionine labeled cellular proteins from human AMA cells (master database) (20). Images can be displayed either in black and white (resembling the original fluorograms) or in color (other images in Fig. 2), depending on the need. As shown in Fig. 2*B*, each polypeptide is assigned a number by the computer, which facilitates the entry and retrieval of qualitative and quantitative information for any given spot in the gel (20). The standard image can be matched automatically by the computer to other standard or reference gels (Fig. 2*C*, matching of AMA cellular proteins [left] to MRC-5 proteins [right]) provided a few landmark spots are given manually as reference (indicated with a + in Fig. 2*C*) to initiate the process.

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[2]Abbreviations: CCD, charge couple device; PCNA, proliferating cell nuclear antigen; HPLC, high performance liquid chromatography.

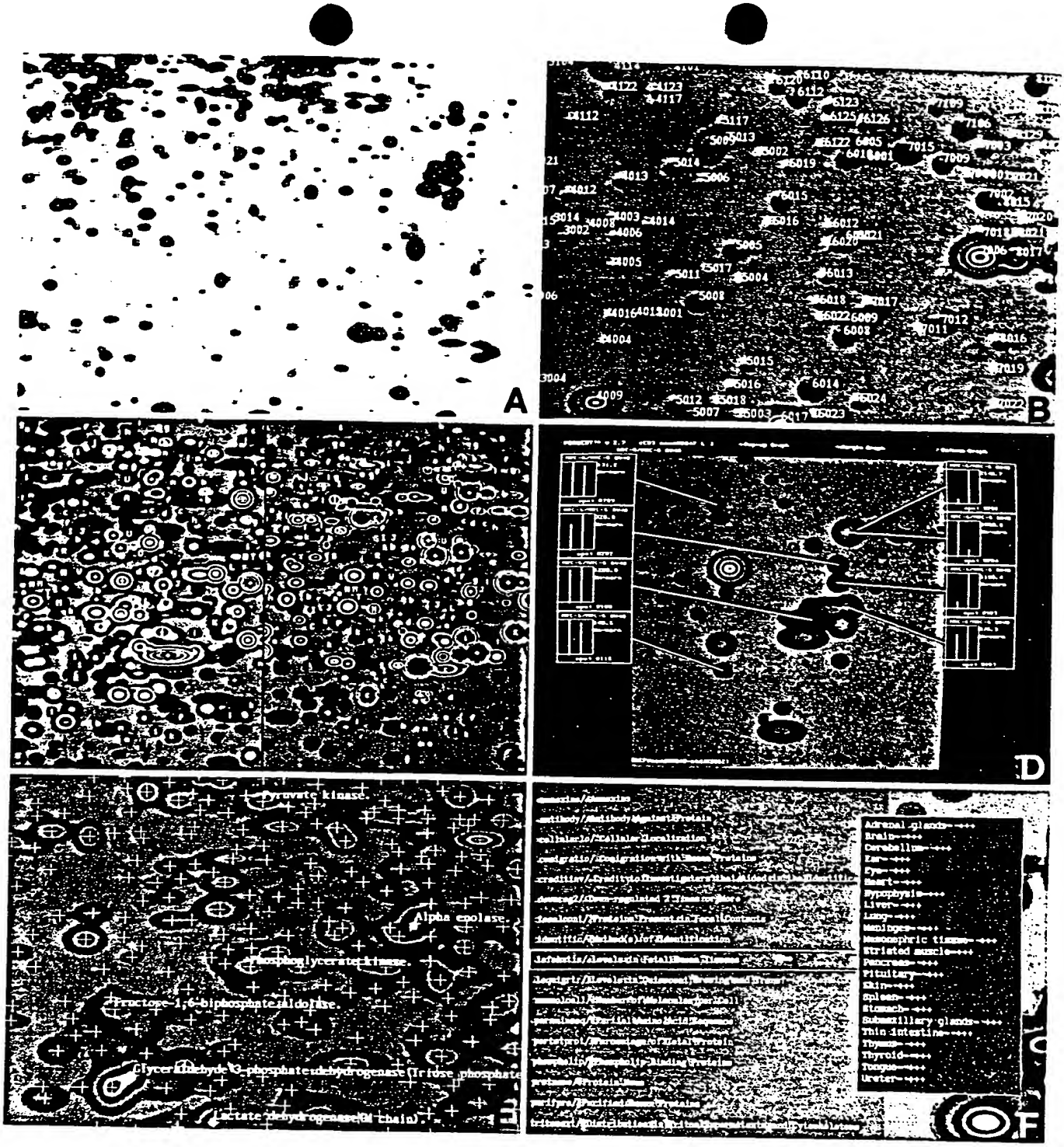
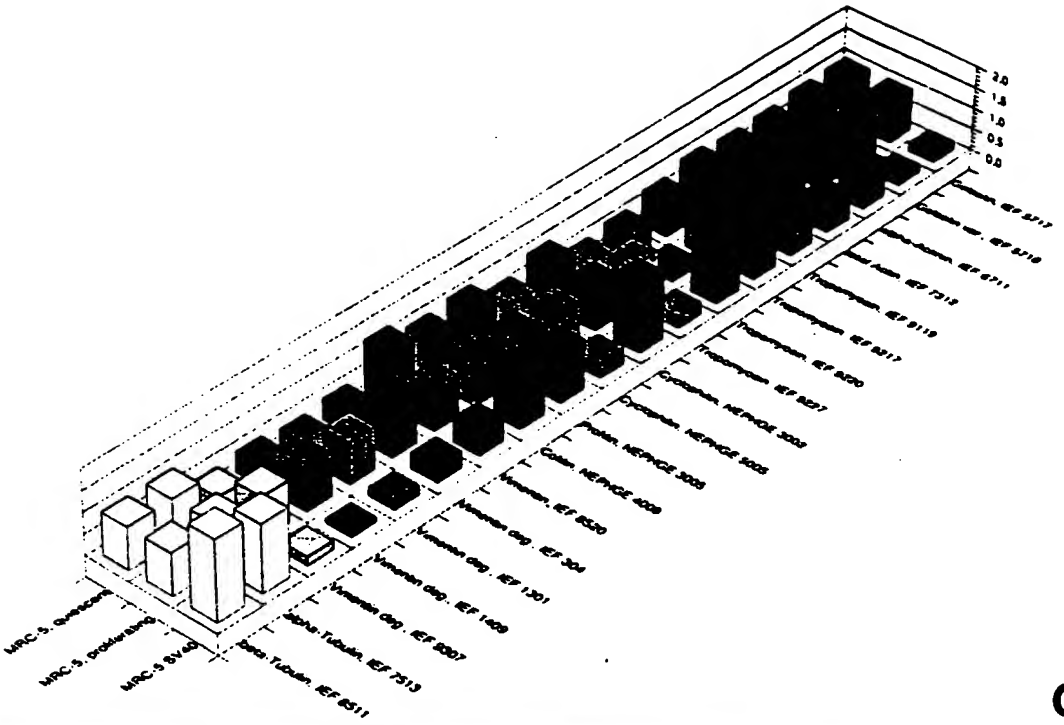


Figure 2. *A*) Synthetic image of a fraction of an IEF gel of the master image of AMA cellular proteins. *B*) As in *A* but showing numbers assigned to each spot. *C*) Comparison of AMA (left) and normal human embryonal lung MRC-5 fibroblasts (right) IEF proteins patterns. Matched proteins are indicated by a + or by the same letters in both gels. Once a protein is matched, information contained in the various categories available in the master AMA database can be transferred. *D*) Synthetic image of a fraction of an IEF fluorogram of [^{35}S]methionine labeled proteins from normal human MRC-5 fibroblasts. The histograms show levels of synthesis of a few proteins in MRC-5 (left bar) and SV40 transformed MRC-5 (right bar) fibroblasts. *E*) Polypeptides that contain information under the category glycolytic pathway. *F*) The function peruse annotation for spot allows the operator to inquire about categories and information available for a given protein. *G*) Relative abundance of cytoskeletal and cytoskeletal-related proteins in quiescent, proliferating, and SV40-transformed MRC-5 fibroblasts. *H*) Polypeptides that contain information under the category partial amino acid sequences.



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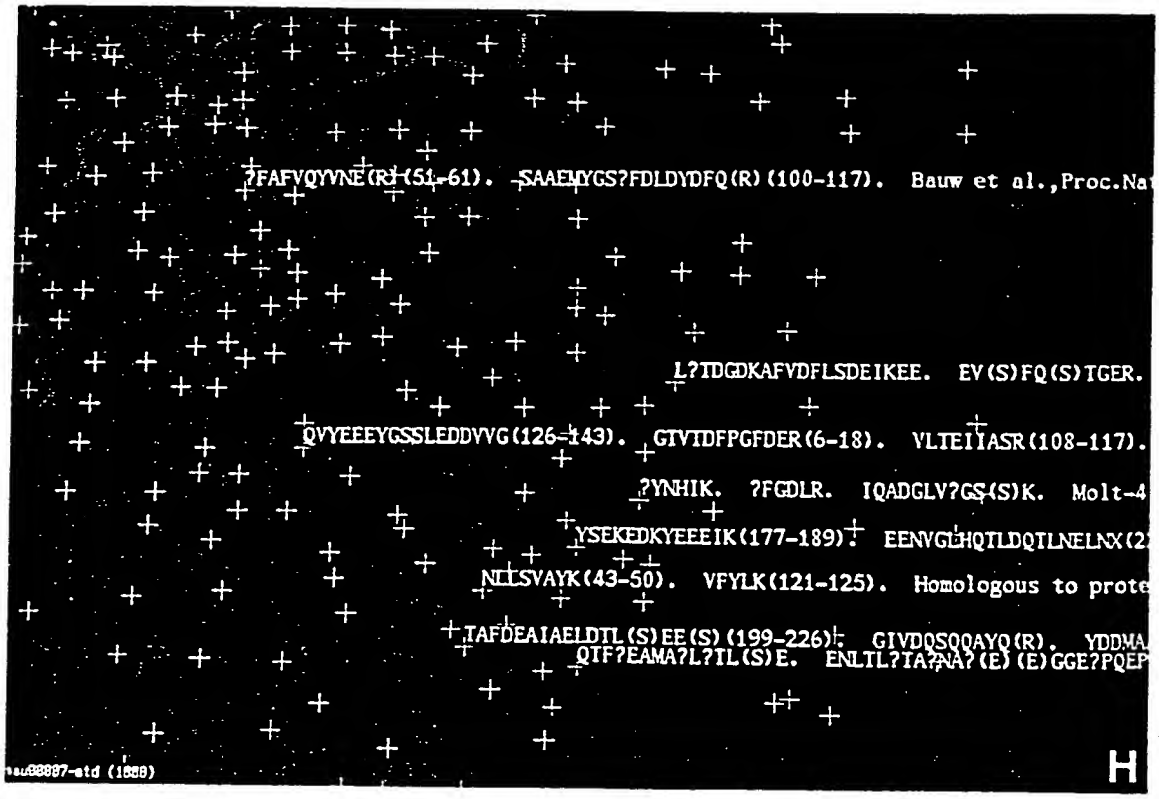

The automatic matching process that has been described in detail by Garrels et al. (12) takes about 5 min. Matched proteins are indicated with the same letters in both gels (Fig. 2C). The usefulness of this function is emphasized by the fact that data accumulated on common household proteins can be easily transferred to any other human cellular cell type whose 2-dimensional gel cellular protein pattern is matched to our standard AMA 2-dimensional gel protein image. Alternatively, if the standard gel is part of a matchset (set of gels in a given experiment) it can be used as a linker gel to compare, for example, the quantitative values of a given protein throughout the experiment (see Fig. 2*D*; levels of some proteins in normal and SV40 transformed human MRC-5 fibroblasts) or with other standard images in different sets of

cross-matched experiments (18, 22).

Once a standard map of a given protein sample is made, one can enter qualitative annotations to make a reference database. Our master 2-dimensional gel database of transformed human amnion cell (AMA) proteins (20) lists 3430 polypeptides of which 2592 correspond to cellular components, having pI's ranging from 4 to 13 and molecular weights between 8.5 and 230 kDa. The most abundant proteins in the database correspond to total actin (3.87% of total protein; about 90 million molecules per cell) while the lesser abundant of the recorded polypeptides are present in the vicinity of 5000 molecules per cell. Some annotation categories we are using to establish the master AMA database include: *1*) protein identification (comigration with purified proteins, 2-dimensional immunoblotting, microsequencing); *2*) amounts (total amounts and levels of synthesis); *3*) subcellular localization (nuclear, cytoskeletal, membrane, membrane receptors, specific organelles, etc.); *4*) antibodies; *5*) posttranslational modifications (phosphorylation, glycosylation, methylation etc.); *6*) microsequencing; *7*) cell cycle specificity (specific variations in levels of synthesis and amount); *8*) regulatory behavior (effect of hormones, growth factors, heat shock, etc.) *9*) rate of synthesis in normal and transformed cells (proliferation sensitive proteins, cell cycle specific proteins, oncogenes, components of the pathway (or pathways) that control cell proliferation); *10*) function (mainly from comigration with proteins of known function); *11*) sets of proteins that are coordinately regulated (hierarchy of controls, differential gene expression in various cells, etc.); *12*) cDNAs (cloned cDNAs); *13*) proteins that are specific to a given disease (systematic comparison of protein patterns of fibroblast proteins from healthy and diseased individuals); *14*) expression and exploitation of transfected cDNAs; *15*) pathways (metabolic, others); *16*) gene localization (genetic and physical); *17*) effect of microinjected antibody on patterns of protein synthesis; and *18*) secreted proteins.

Information entered for any spot in a given annotation category can be easily retrieved by asking the computer to display the information on the color screen. For example, Fig. 2*E* shows a synthetic image of a NEPHGE gel (master AMA database) displaying the information contained under the entry glycolytic pathway. Alternatively, one can use the function peruse annotations for spot to directly ask the computer to list all the entries available for a particular protein. By clicking the mouse in a given entry (in this case, presence in fetal human tissues) it is possible to take a quick look at the information in that particular entry (Fig. 2*F*).

A major obstacle encountered in building comprehensive 2-dimensional gel protein databases is identifying the large number of proteins separated by this technology. In our databases (20, 21), known proteins are identified by one or a combination of the following procedures: *1*) comigration with known proteins, *2*) 2-dimensional gel immunoblotting using specific antibodies, and *3*) microsequencing of Coomassie Brillant Blue stained human proteins recovered from dried 2-dimensional gels (see next section). Protein identification by means of microsequencing may be difficult, as individual protein members of families with short peptide differences may escape detection. In the gene-protein database of *E. coli* K-12 (14, 23), another major 2-dimensional gel database available at present, proteins are being identified by a wider range of tests that include comigration with purified proteins; genetic criterion (deletion, insertion, frameshift, nonsense, missense, regulatory), plasmid-bearing strains and in vitro synthesis of protein; selective labeling (methylation, phosphorylation); peptide map similarity; and physiological criterion and selective derivatization.

So far we have received nearly 550 antibodies from laboratories all over the world and these are being systematically tested by 2-dimensional gel immunoblotting for antigen determination. Similarly, purified proteins and organelles provided by several laboratories have greatly aided identification of unknown proteins (20, 21). We routinely request antibodies and protein samples and promise the donors to make available all the information we may have accumulated on that particular protein. For example, **Table 1** lists entries available for Lipocortin V (IEF SSP 8216), also known as annexin V, VAC-α, endonexin II, renocortin, chromobindin-5', anticoagulant protein, PAP-I, γ-calcimedin, IBC, calphobindin, and anchorin CII.

As mentioned previously, one distinct advantage of 2-dimensional gel electrophoresis is the possibility of studying quantitative variations in cellular protein patterns that may lead to identification of groups of proteins that are expressed coordinately during a given biological process. Quantitation, however, is not an easy task as reflected by the lack of published data on global cellular protein patterns. We believe this is partly due to difficulties in obtaining sets of gels that are suitable for computer analysis (streaking, material remaining at the origin, etc.) as well as to limitations (laborious editing time, need of calibration strips to merge images, limited dynamic range, etc.) in the computer analysis systems available at the moment. Perhaps the most advanced quantitative studies published so far using computer analysis have been carried out by Garrels and coworkers (18, 22). In particular, these investigators have established a quantitative rat protein database (18, 22) designed to study growth control (proliferation, growth inhibitors, and stimulation) and transformation in well-defined groups of cell lines obtained by transformation of rat REF52 cells with SV40, adenovirus, and the Kirsten murine sarcoma virus. These studies have revealed clusters of proteins induced or repressed during growth to confluence as well as groups of transformation-sensitive proteins that respond in a differential fashion to transformation by DNA and RNA viruses. A most interesting feature of this quantitative database is the discovery of a group of coregulated proteins that show similar expression patterns as the cell cycle-regulated DNA replication protein known as proliferating cell nuclear antigen (PCNA)/cyclin (45).

In our human databases, most quantitations have been carried out by estimating the radioactivity contained in the polypeptides by direct counting of the gel pieces in a scintillation counter (20, 21). Up to 700 proteins can be cut out through appropriate exposed films in a period of time comparable to that required for editing a synthetic image. Manual quantitation of this large number of spots is difficult without the assistance of a master reference image and a numbering system that can be used to identify the spots. Using this approach, we have recorded quantitative changes in the relative abundance of 592 [^{35}S]methionine-labeled proteins synthesized by quiescent, proliferating, and SV40 transformed human embryonic lung MRC-5 fibroblasts (21). Some data concerning cytoskeletal and cytoskeletal-related proteins are presented in Fig. 2G. Our studies as well as those of Garrels and co-workers (18, 22) may in the long run help define patterns of gene expression that are characteristic of the transformed state.

OTHER 2-DIMENSIONAL GEL PROTEIN DATABASES

As mentioned previously there are other 2-dimensional gel databases available in computer form that have been pub-

TABLE 1. *Some entries for lipocortin V in the human AMA 2-dimensional gel protein database*

Entries for lipocortin V (IEF SSP 8216)	Information entered
1. Protein name	Lipocortin V, renocortin, chromobindin-5', endonexin I, anticoagulant protein, PAP-I, VAC-α, 35-γ-calcimedin, IBC, calphobindin I, anchorin CII, annexin V
2. Percentage of total protein	0.110% (about 2,800,000 molecules per cell)
3. Apparent molecular weight (*mr*)	33.3 kDa
4. Isoelectric point (pI)	4.76
5. Method (or methods) of identification	Microsequencing, 2-dimensional immunoblotting, Comigration
6. Credit to investigators that aided in identification	G. Bauw, J. Vandekerckhove, and colleagues, Rijksuniversiteit Gent; B. Pepinsky, BIOGEN, Cambridge; N.G. Ahn, University of Washington
7. Antibody against protein	Polyclonal (rabbit, antibody no. 20), B. Pepinsky, BIOGEN, Cambridge
8. Comigration with human proteins	Lipocortin V, N.G. Ahn, Howard Hughes Medical Institute, Washington University
9. Cellular localization	Subcortical membrane
10. Calcium/phospholipid-dependent membrane proteins	Lipocortin V
11. Function	Regulation of various aspects of inflammation, immune response, blood coagulation and differentiation
12. Partial amino acid sequence	GTVTDFPGFDER (7–18), VLTEIIASR (109–117), QVYEEEYGSSLEDDVVG (127–143), ?GTDEEKFITIFGT(R) (187–201)
13. cDNA sequence	Known, R. Blake et al., *J. Biol. Chem.* 263, 10799–10811; 1988 (pI = 4.76 from translated sequence)
14. Levels in fetal human tissues	Adrenal glands = + + +; brain = + + +; cerebellum = + + +; ear = + + +; eye = + + +; heart = + + +; hypophysis = + + +; liver = + + +; lung = + + +; meninges = + + +; mesonephric tissue = + + +; striated muscle = + + +; pancreas = + + +; skin = + + +; spleen = + + +; stomach = + + +; submandibular gland = + + +; small intestine = + + +; thymus = + + +; thyroid gland = + + +; tongue = + + +; ureter = + + +
15. Levels in quiescent, proliferating, and transformed MRC-5 fibroblasts	Q (quiescent) = 1.1; P (proliferating) = 1.0; T (SV40 transformed) = 0.3
16. Distribution in Triton supernatant and cytoskeletons	Mainly supernatant

lished in extenso: these correspond to the *E. coli* K-12 protein-gene database (14, 23) and to the rat REF52 database (18, 22).

The *E. coli* K-12 cellular protein-gene database is perhaps the most complete of all databases reported so far and eventually it should trace each protein back to its structural gene. Information contained in this database includes: gene/protein name (protein name, EC number, gene name); 2-dimensional gel spot designations (x-y coordinates from reference gels, alphanumeric designation); genetic information (linkage map location, physical map location, Genebank code, sequence reference, location on Kohara clones); biochemical information (molecular weight, pI, number of residues of each amino acid, mole percent of each amino acid, total number of amino acids in a polypeptide), and regulatory information (cellular level of protein in different media and different temperature, member of regulon, member of stimulon). Major advances of this database are envisaged in the future in view of the eminent sequencing of the whole *E. coli* genome as well as the development of improved methods to express cloned genes.

The rat REF52 2-dimensional gel protein database lists about 1600 proteins that have been recorded using the QUEST analysis system (18, 22). Included in this quantitative database are *1*) protein names (cytoskeletal and heat shock proteins as well as various nuclear, mitochondrial, and cytoplasmic proteins), *2*) annotations (subcellular localization, modification, recognition by specific antibodies, coprecipitation, NH_2-terminal sequence, cross-reference to protein sequence information and references to the literature), *3*) protein sets (cytoskeletal proteins, phosphoproteins, sets of proteins with PCNA/cyclin-like properties, etc.) and *4*) general quantitative data (protein synthesis during growth of normal REF52 cells to confluence and quiescence, and after restimulation of growth-inhibited cells).

In addition to the 2-dimensional gel databases mentioned so far there are several smaller cellular databases being established in human (normal human diploid fibroblasts, lym-

phocytes, leukocytes, leukemic cells) mouse (NIH/3T3 cells, T lymphocytes), *Aplysia*, yeast (*Saccharomyces cerevisae*), plants (wheat, barley, sorghum), and *Euglena*. Databases of tissue protein, (brain, whole mouse, liver) and body fluid proteins (plasma proteins, cerebrospinal fluid, urine, and milk) are being established in several laboratories. The reader is directed to the review by Celis et al. (4) for details and references concerning these databases.

MICROSEQUENCING HAS ADDED A NEW DIMENSION TO COMPREHENSIVE 2-DIMENSIONAL GEL DATABASES: A DIRECT LINK BETWEEN PROTEINS AND GENES

The development of highly sensitive amino acid gas-phase or liquid-phase sequenators (24), together with the establishment of efficient protein and peptide sample preparation methods, has opened the possibility to perform a systematic sequence analysis of proteins resolved by 2-dimensional gel electrophoresis. Indeed, generated pieces of protein sequences can be used to search for protein identity (comparison with available sequences stored in databanks) as well as for preparing specific DNA probes for cloning of as yet uncharacterized proteins (Fig. 1). In addition, partial protein sequences can be stored in 2-dimensional gel databases (for example, see Fig. 2*H*) and offer a unique link between proteins and genes (Fig. 1).

In the early 1970s gel electrophoresis was used to purify proteins for sequencing purposes (reviewed by Weber and Osborn in ref 25). Proteins were recovered by diffusion and sequenced by the manual dansyl-Edman degradation at the nanomole level. This technique was further refined by using electro-elution to recover proteins and by miniaturizing the system (26). This method has been used extensively, but showed increasing drawbacks (low yields, protein samples contaminated by free amino acids, and NH_2-terminal blocking) as the amounts of handled protein gradually became smaller (e.g., at the 10 picomol level).

Most of the problems referred to above have been minimized with the introduction of protein-electroblotting procedures (27–32). When proteins are blotted on chemically inert membranes, it is possible to sequence the immobilized proteins directly without additional manipulations. Thus, depending on the amount of bound protein and its nature, this direct sequencing procedure generally yields NH_2-terminal sequences containing 10–40 residues. As such, this technique was used to identify, by their NH_2-terminal sequences, differentially expressed major proteins from total cellular extracts separated on 2-dimensional gels. A major difficulty encountered in this procedure is the occurrence of frequent artefactual blockage of the proteins. Several studies suggest that this phenomenon is mainly due to reaction with contaminants (particularly unpolymerized acrylamide present in the gel) and to a high dilution of the protein (low concentration of the protein per unit membrane surface). In addition to this primarily technical problem, many proteins are blocked in vivo by acylation or by a pyrrolidon carboxylic acid cap.

The problem of partial or complete NH_2-terminal blockage can be circumvented by generating internal amino acid sequences. This is achieved by fragmenting the protein present in the gel (gel in situ cleavage) or by cleaving it while bound to the membrane (membrane in situ cleavage) (33–35). In both cases, proteins are either cleaved in a restricted way (e.g., by limited enzymatic digestion or by using restriction chemical cleavage conditions) or fragmented into smaller peptides.

Of the different combinations examined, we had good results by using exhaustive proteolytic digestion on membrane-immobilized proteins. This method has been described for Ponceau red-stained proteins on nitrocellulose blots (34), for Amido-black-stained Immobilon-bound proteins, and for fluorescamine-detected proteins on glass fiber membranes (35). The proteases used (trypsin, chymotrypsin, or pepsin) cleave at multiple sites, generating small peptides that elute from the blot into the digestion buffer from which they are purified by reversed-phase high performance liquid chromatography (HPLC) before being sequenced individually. Although each of these manipulations could be expected to result in a reduced yield of final sequence information, we were surprised that the peptides could be sequenced with high efficiency. In our hands, this approach could be routinely applied to gel-purified proteins available in amounts ranging from 5 to 10 μg, and often yielded sequence information covering more than 30% of the total protein. As membrane-immobilized proteins are not homogeneously digested, but rather show protease sensitivity next to resistant regions, the number of peptides generated is much lower than expected from the number of potential cleavage sites. Consequently, HPLC peptide chromatograms are less complex and most peptides can be recovered in pure form.

As only limited amounts of a protein mixture can be loaded on a 2-dimensional gel, proteins of interest are often obtained in yields insufficient for the currently available sequencing technology. More material can be obtained by enriching for a certain subcellular fraction (purified cell organelles) or by exploiting affinity (dyes, metals, drugs, etc) or hydrophobic properties of proteins before gel analysis. All of the sequencing results accumulated so far in the human protein database (20) (a few are shown in Fig. 2*H*) have been obtained from analysis of protein spots collected from 2-dimensional gels that had been stained with Coomassie blue according to standard procedures and dried for storage. Proteins are recovered from the collected gel pieces by a protein-elution-concentration device, combined with gel electrophoresis and electroblotting. Details of this technique have been reported in a previous communication (42) and a brief outline is given below.

Combined gel pieces are allowed to swell in gel sample buffer (a total volume of 1.5 ml). The gel pieces combined with the supernatant are then collected into a large slot made in a new gel. The slot is further filled with Sephadex G-10 equilibrated in gel sample buffer. During consecutive gel electrophoresis, most of the electrical current passes on the side of the slot instead of passing through the slot. This results in both a vertical stacking and horizontal contraction of the protein band. With this device the protein is efficiently eluted from the gel pieces and concentrated from a large volume into a narrow spot. The highly concentrated (about 5 mm^2) protein spot is then electroblotted on PVDF-membranes, stained with Amido black, and in situ digested with trypsin. The peptides generated during digestion elute from the membrane into the supernatant, and can be separated by narrow bore reversed-phase HPLC and collected individually for sequence analysis.

Using this and previous procedures (37, 39, 42), we have so far analyzed 70 protein spots collected from 2-dimensional gels (20, and unpublished observations) (see for example Fig. 2*H*). The sequence information amounts to 2100 allocated residues corresponding to an average of 30 residues per protein spot. So far we have made cDNAs of many of the unknown proteins that have been microsequenced, and a substantial number has been cloned and sequenced. All available information indicates that it may be possible to obtain partial sequence information from most of

the proteins that can be visualized by Coomassie Brillant Blue staining.

Partial protein sequences are stored in the database as displayed in Fig. 2*H*, and it should be possible in the near future to interface this information with forthcoming DNA sequence data from the human genome project. In the long run, as the human genome sequences become available it will be possible to assign partial protein sequences to genes for which the full DNA sequence and chromosomal location are known (Fig. 1).

SUMMARY

The studies presented in this brief review are intended to demonstrate the usefulness of computer-aided 2-dimensional gel electrophoresis and microsequencing to analyze cellular protein patterns, and to link protein and DNA information. As more information is gathered worldwide, comprehensive databases will depict an integrated picture of the expression levels and properties of the thousands of proteins that orchestrate most cellular functions.

Clearly, databases allow easy access to a large body of data and provide an efficient medium to communicate standardized protein information. In the future, databases will foster a wide variety of biological information that can be used to support collaborative research projects in basic and applied biology as well as in clinical research (2, 5, 46). Once a protein is identified in a particular database all the information gathered on it can be made available to the scientist. However, many problems must be solved before protein databases become of general use to the scientific community. A most urgent one is to promote standardization of the gel running conditions so that data produced in a given laboratory may be used worldwide. Surprisingly, the gel running technology as it stands today is still a craftmanship art.

Finally, comprehensive, computerized databases of proteins, together with recently developed techniques to microsequence proteins, offer a new dimension to the study of genome organization and function (Fig. 1). In particular, human protein databases may become increasingly important in view of the concerted effort to map and sequence the entire human genome. This formidable task is expected to dominate biological research in the next decades. FJ

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Nonenzymatic extraction of cells from clinical tumor material for analysis of gene expression by two-dimensional polyacrylamide gel electrophoresis

We have compared different methods of preparation of malignant cells for two-dimensional electrophoresis (2-DE). We found all methods using fresh tissue to be superior compared to methods using frozen tissue. Our results indicate that nonenzymatic methods of preparation of tumor cells, including fine needle aspiration, scraping and squeezing, have advantages over methods using enzymatic extraction of cells. Nonenzymatic methods are rapid, appear to reduce loss of high molecular protein species, and alleviate the necessity of separating viable and nonviable cells by Percoll gradient centrifugation. Using these techniques, high-quality 2-DE maps were derived from tumors of the lung and breast. In the resulting polypeptide patterns, heat shock proteins, non-muscle tropomyosins and intermediate filament were identified. We conclude that nonenzymatic extraction of malignant cells from fresh tumor tissue improves the possibilities that these techniques may be useful in clinical diagnosis.

1 Introduction

Tumors may develop by a number of different mechanisms in any given cell type. At the time of diagnosis, tumors will have progressed along different pathways to various stages of malignancy. To provide a basis for individual therapy it is of importance to examine specific properties of the tumor cell population in each patient. A large number of different markers have been described in order to increase the diagnostic accuracy. It is likely that a combination of serveral markers is needed in the future in order to reflect different properties of the tumor. One important method for the resolution of a large number of potential markers is two-dimensional electrophoresis (2-DE). Extensive efforts are being made in identifying various polypeptides separated by 2-DE and to characterize how the expression of these polypeptides is affected by the response to cellular transformation and various culture conditions [1,2]. It would be of value to transfer this information to 2-DE separations of polypeptides from tumor tissue samples. However, one prerequisite is that the quality of the 2-DE gels from tumor samples is comparable in quality with 2-DE gels from samples of cultured cells.

Frozen tumor tissues are commonly used for various biochemical assessments. However, if such samples are analyzed by 2-D polyacrylamide gel electrophoresis (PAGE), the polypeptide patterns are obscured by contamination of serum- and connective tissue proteins. Such nontumor-cell-related variations represent serious problems in the interpretation and inter-patient comparison of 2-DE patterns [3]. 2-DE patterns of cells prepared from fresh tumor material were analyzed after enzymatic extraction of tumor cells [4, 5] or after culturing tumor fragments in medium containing radioactive amino acids [6]. These procedures may, however, lead to alterations in the gene expression/polypeptide patterns. We are only aware of one study where nonenzymatic extraction of cells from fresh tumor tissue (prostate cancer) was used to prepare samples for 2-D PAGE [4]. We have examined enzymatic extraction and various nonenzymatic preparation techniques, including fine needle aspiration, for the preparation of cells from fresh tumor tissues. We describe nonenzymatic extraction procedures that are rapid, lead to high-quality 2-DE patterns, and that alleviate the necessity to purify tumor cell populations from dead cells.

2 Materials and methods

2.1 Cell cultures and samples used for spot identification

A rat embryonal fibroblast cell line, WT2 (a kind gift from Dr. J. I. Garrels and Dr. S. Pattersson) was used for the identification of a number of heat shock and structural proteins. Human normal diploid lung fibroblasts, WI38, human epithelial breast carcinoma cells, MDA-231 and MCF-7 were purchased from ATCC and grown as recommended. Polypeptides prepared from a leukemia type pre-B-ALL were separated by 2-DE. The 2-DE map was then analyzed by Dr. S. M. Hanash (University of Michigan, Ann Arbor, USA).

2.2 Tumor tissues samples

In this study, 2-DE maps from seven tumors were used as representative illustrations: two adenocarcinoma of the lung (LA, and LB, mucinous, both cases intermediate grade of differentiation), one sqamous carcinoma of the lung (LS), one carcinoid-like breast cancer (BC), one microfolliculary adenoma (highly differentiated) of the thyroid (TA), one highly differentiated hyperneph-

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Abbreviations: **2-DE**, Two-dimensional polyacrylamide gel electrophoresis; **IEF**, isoelectric focusing; **LDH**, lactate dehydrogenase; **NP-40**, Nonidet P-40; **PBS**, phosphate buffered saline; **PCNA**, proliferating cell nuclear antigen; **PIH**, protease inhibitors; **PMSF**, phenylmethyl sulfonyl fluoride; **SDS**, sodium dodecyl sulfate; **WW**, wet weight

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roma, a tumor of the kidney (KH), and finally one case of poorly differentiated corpus carcinoma (CP).

2.3 Preparation of cultured cells

The cell monolayers were washed twice in phosphate buffered saline (PBS) and then scraped off in ice-cold PBS including protease inhibitors (PIH), phenylmethylsulfonyl fluoride (PMSF) 0.2 mM and 0.83 mM benzamidine pelleted at 660 × *g*, 3 min (+4°C) and washed one time before final centrifugation at 2700 × *g*, 5 min. The wet weight of the cell pellet was recorded and the cells were stored at −80°C until further processing.

2.4 Preparation of tumor tissue samples

2.4.1 General remarks

Macroscopically representative and non-necrotic tumor tissues were selected within 20 min after resection. Parallel samples were routinely prepared for cytology. The samples were processed as rapidly as possible on ice or at +4°C and in the presence of PIH. Cells were stained with DiffQuick (Baxter) and usually examined at three different occasions during the preparation procedure: (i) cytology sample, (ii) extracted cells and (iii) cells after percoll gradient centrifugation.

2.4.2 Specimen acquisition

The strategy of sample preparation is shown in Fig. 1. Tumor tissue cell samples were usually obtained by fine needle aspiration (NA) using a 0.7 mm needle. The syringe was filled with 1–2 mL of ice-cold culture medium/PIH. We found that if a tumor appeared to be very fibrous it is difficult to extract enough cells for 2-DE analysis. In these cases, two alternative techniques were examined. (i) The tumor was cut in the middle and the fresh surface scraped (SC) by a scalpel. The cell-rich material was then transferred to ice-cold culture medium (L15 with 5% fetal calf serum)/PIH. (ii) A part of the tumor sample was placed in culture medium on ice for further processing at the laboratory in the following way: the material was cut into very small fragments on a pre-cooled dissection plate and transferred to a small glass chamber with a 0.7 mm metal net 5 mm above the bottom of the chamber. Medium /PIH was added to cover the sample (8 mL) which was gently squeezed (SQ) towards the net in order to release and wash out cells. NA and SC were also compared with an enzymatic extraction (EE) procedure described previously [5]: Briefly, thin slices of tissue were incubated with collagenase (1 mg/mL) and elastase (2 mg/mL) in medium for 1 h at 37°C. Extracted cells from every sample were then subjected to percoll gradient centrifugation (Section 3.2.3).

2.4.3 Separation of cells by Percoll gradient centrifugation

The cell suspension was filtered through two nylon mesh filters, (i) 250 µm and (ii) 100 µm and then centrifuged at 660 × *g* for 3 min. The cell pellet was resuspended carefully in medium, using a syringe and loaded onto a two-step discontinuous Percoll/PBS gradient, 20.4% (density = 1.03 g/mL) and 54.7% (density = 1.07 g/mL), and centrifuged at 1000 × *g* for 15 min. In this system, dead cells stay on the top, viable cells sediment to the interphase and erythrocytes sediment to the bottom. The viability of cells in the top fraction and interphase was checked by the trypan blue exclusion test. The interphase cell layer (> 90% viability) was collected and washed one time in a large volume PBS/PIH (centrifuged at 800 × *g* for 3 min). Finally, the cells were resuspended in 1.4 mL PBS and pelleted at 2700 × *g* for 5 min. The wet weight (WW) was recorded and the pellet was then stored at −80°C.

2.4.4 Final preparation of cells for 2-D PAGE analysis

From this point, cultured cell samples were treated in the same way as tumor cell samples: Each cell pellet was thawed on ice and resuspended in 1.89 µL mQ water per mg WW (= 1.89 × WW) µL. The suspension was frozen and thawed 4–5 × to break the cells [7]. A volume of (0.089 × WW) µL 10% sodium dodecyl sulfate (SDS), including 33.3% mercaptoethanol, was mixed with the sample and incubated 5 min on ice with (0.329 × WW) µL of a solution of DNase I (0.144 mg/mL 20 mM Tris-HCl with 2 mM $CaCl_2 \times 2H_2O$, pH 8.8) and RNase A (0.0718 mg/mL Tris) [8,9]. The sample was frozen and lyophilized. Sample buffer [10] including

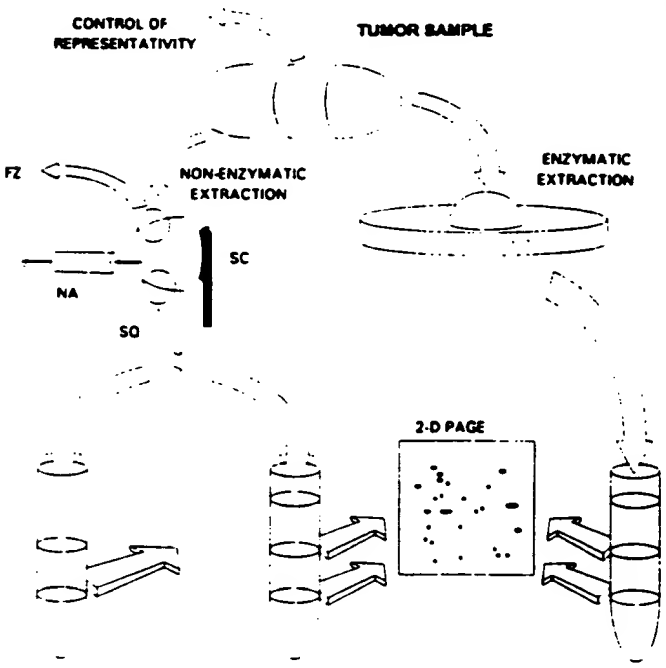

Figure 1. Experimental flow chart showing main steps of the preparation procedures. The abbreviations used for nonenzymatic extraction procedures are: FZ: frozen sample preparation; NA, needle aspiration; SC, scraped; and SQ, squeezed sample. Extracted cells are then loaded as a suspension (top volume of each tube) onto either 1.07 g/mL Percoll (left), or a discontinuous Percoll gradient from the nonenzymatic extraction (middle), or from enzymatic extraction (right). Cellular top- and interphase fractions are then used for 2-DE. For details see Section 2.

PMSF (0.2 mM, EDTA (1.0 mM), 0.5% Nonidet P-40 (NP-40), and 3-[3-cholamido propyl)-dimethylammonio]-1-propane sulfonate (CHAPS; 25 mM) was added carefully, mixed for 2.5 h and centrifuged for 15 min at 10000 rpm to remove any insoluble material. Duplicate or triplicate samples were taken for protein determination [11]. Samples were stored at −80°C prior to isoelectric focusing (IEF).

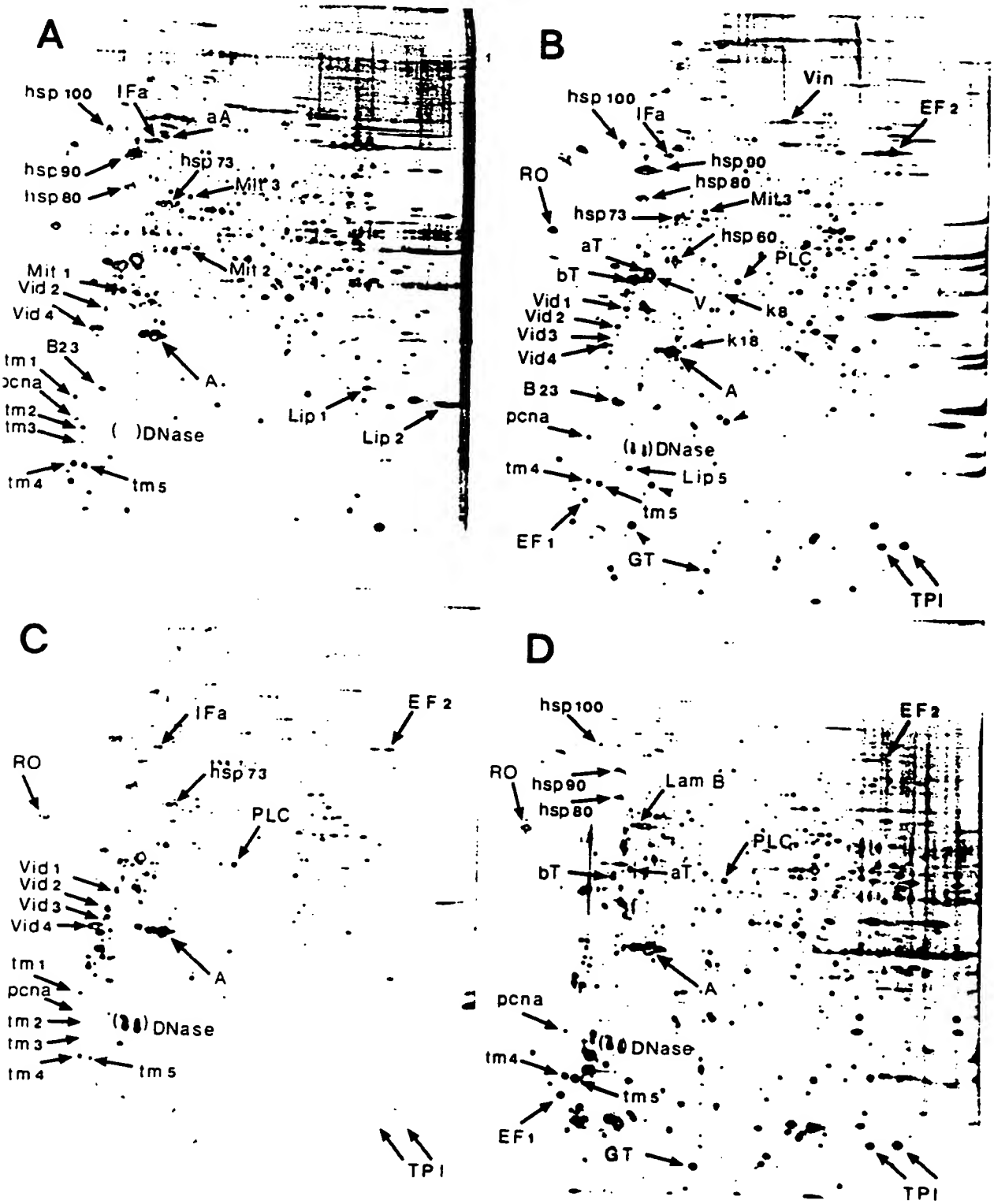

Figure 2. 2-DE analysis of samples from three cell lines and one leukemia used for the identification of polypeptides: (A) WT2; (B) MDA-231, arrowheads mark some low molecular weight cytosolic polypeptides; (C) WI38 and (D) pre B-All. The abbreviations for identified spots are explained in Table 1.

2.4.5 Preparation of frozen tumor tissue

The technique has been described previously [3,12]. Briefly, the sample is moarted frozen to a fine powder, homogenized, lyophilized and solubilized in sample buffer.

2.4.6 Control of representativity

The tumors were examined routinely by experienced pathologists and smears or imprints from the samples were also assessed for cytometric DNA content by microspectrophotometry.

2.5 2-D PAGE

2-D PAGE was performed as described [8,10] except for the following details. The glass tubes for IEF, 1.2 × 200 mm, contained 2.0% Resolyte, pH 4–8 (BDH) and were cast to a height of 180 mm. A stock solution of acrylamide (Serva) and *N,N'*-methylenebisacrylamide (16.7:1 for IEF and 37.5:1 for the second dimension) was deionized by mixing with 5% w/v Duolite MB 5313 mixed-resin ion exchanger (BDH) for 30 min, filtered (with a 0.22 µm nitrocellulose filter) and stored at −70°C. *N,N'*-Methylenebisacrylamide, *N,N,N',N'*-tetramethylethylenediamine (TEMED) and ammonium persulfate were purchased from Bio-Rad. IEF tubes were prefocused at 200 V in 60 min. To each tube a sample corresponding to 20–40 µg protein was applied and focused for 14.5 h at 800 V and finally 1.0 h at 1000 V using a Protean II cell (Bio-Rad) and Model 1000/500 Power Supply (Bio-Rad). The tube gels were finally extruded into 1.25 mL equilibration buffer, containing 60 mM Tris, pH 6.8 (2% SDS, 100 mM dithiothreitol and 10% glycerol), frozen on dry ice and stored at −70°C. The second dimension (1.0 × 180 × 90 mm) of the acrylamide concentration was 10% T, and the gel contained 376 mM Tris, pH 8.8, and 0.1% SDS. IEF gels were applied on top of the slab gel, sealed with 0.5% agarose containing electrophoresis running buffer (60 mM Tris-base, 0.2 M glycine and 0.1% SDS) and electrophoresed with 10–11 mA per gel (constant current) at +10°C. Six gels were run together in a Protean II xi 2-D Multi-Cell (Bio-Rad). Proteins were visualized by silver staining and photographed with the acidic side to the left [13,14].

2.6 Identification of polypeptides

Vimentin and vimentin-derived polypeptides were identified by extraction of an MDA-231 cell lysate with 0.6 M KCl/0.5% NP-40 [15]. Tropomyosins were extracted from MDA-231 and WI38 cell lysates [16], and cytokeratins were extracted from MDA-231 and MCF-7 cell lysates [17]. The patterns were compared with published maps [19–21]. Proliferating cell nuclear antigen (PCNA) was identified by immunoblotting (PC10 mAB, Dakopatt) using a semidry system (Multiphor II Nova Blot, Pharmacia-LKB Biotechnology AB) and enhanced chemoluminescence (ECL) detection (Amersham).

3 Results

3.1 2–DE of samples prepared from normal and tumorigenic cultured cells

The object of this study was to develop methods for preparation of 2-DE maps from human tumor tissue which have the same high resolution as those obtained from cultured cells. Shown in Fig. 2 are high resolution 2-DE gels prepared from cultured cells and one leukemia: SV40 transformed embryonal rat fibroblasts WT2 (Fig. 2a); human MDA-231 breast carcinoma cells (Fig. 2b); human WI38 fibroblasts (Fig. 2c) and human pre B-ALL

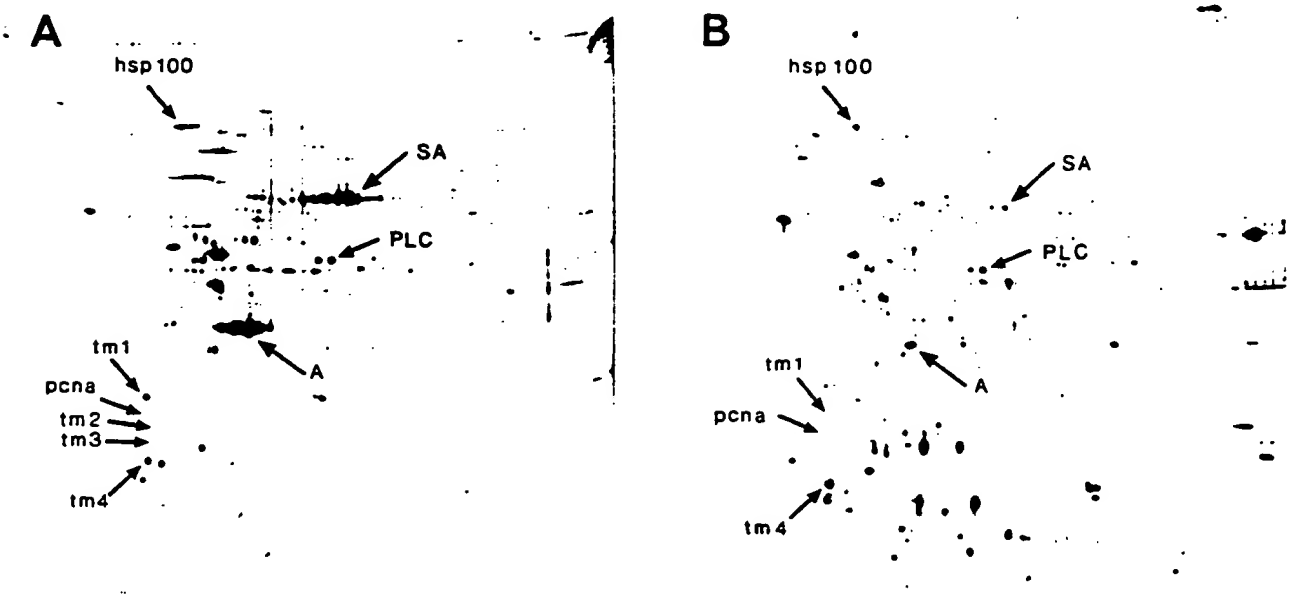

Figure 3. 2-DE analysis of a case of lung adenocarcinoma (LA). Comparison of 2-DE gel quality between (A) frozen and (B) fresh (needle aspiration) tissue preparation.

cells (Fig. 2d). Polypeptides were identified through a laboratory exchange of cell samples/2-DE maps and through 2-DE analysis of purified proteins (Table 1).

3.2 Preparation of samples from solid tumors

3.2.1 Fresh *versus* frozen tissue

An adenocarcinoma of the lung (LA) was prepared for 2-DE by conventional methods using frozen material (Fig. 3a). There are several possibilities for the poor resolution using frozen tissue, including the presence of high molecular weight protein aggregates. Filtering extracts through 0.1 μm filters (Durapore, Millipore) resulted in a slightly improved resolution (not shown). When fresh tumor tissue from tumor LA was used for sample preparation, using fine needle aspiration to collect the cells, the resolution was considerably improved (Fig. 3b). The use of fresh tissue resulted in a general increase in resolution, which was most pronounced in the 50–100 kDa molecular mass range. A number of differences in the protein profiles of the gels in Figs. 3a and 3b can be observed, some of which are indicated in the figures. The decrease in serum albumin in Fig. 3b is likely to result from loss of serum proteins occurring when cells were pelleted after aspiration. Other differences, such as the decreased level of transformation-sensitive tropomyosins (TM1-TM3), may result from enrichment of tumor cells in the sample of Fig. 3b. Fine needle aspiration, a well-established technique in cytology, extracts mainly tumor cells because of decreased intercellular adhesiveness of neoplastic cells as compared to normal tissue. Microscopic examination of Diff-Quick-stained extracted cells from case LA revealed almost 100% tumor cells, whereas the whole tissue extract contained approximately 60% tumor cells.

Table 1. Names and abbreviations for identified spots

Spot	Name	Basis for identification
A	Actins	a
aA	*alpha*-Actinin	a
B23	Protein B23 /Numatrin	a
EF2	Elongation factor 2	a
EF1	Elongation factor 1 β	a
GT	Glutathione-S-transpherase (*pi*	a
hsp60	Heat shock protein 60	a
hsp73	Heat shock protein 73	a
hsp80	Heat shock protein 80, GRP78, BIP	a
hsp90	Heat shock protein 90	a
hsp100	Heat shock protein 100, Endoplasmin	a
IFa	Intermediary filament associated	a
k8	Cytokeratin 8	b and a
LamB	Lamin B	a
Lip1	Lipocortin I	a
Lip2	Lipocortin II	a
Lip5	Lipocortin V	a
Mit1	Mitcon 1/β – F1 ATPase	a
Mit2	Mitcon 2	a
Mit3	Mitcon 3	a
MRP	Mucine Related Polypeptides	–
pcna	Ploliferating cell nuclear antigen	c and a
PLC	Phospholipase C (1)	a
RO	RO/SS-A antigen	a
SA	Serum Albumin	b and a
aT	*alpha*-Tubulin	a
bT	*betha*-Tubulin	a
tm1	Non-muscle tropomyosin isoform 1	b and a
tm2	Non-muscle tropomyosin isoferm 2	b and a
tm3	Non-muscle tropomyosin isoferm 3	b and a
tm4	Non-muscle tropomyosin isoform 4	b and a
tm5	Non-muscle tropomyosin isoform 5	b and a
TPI	Triose phosphate isomerase	a
V	Vimentin	b and a
Vid1	Vimentin derived protein	b and a
Vid2	Vimentin derived protein	b and a
Vid3	Vimentin derived protein	b and a
Vid4	Vimentin derived protein	b and a
Vin	Vinculin	a

a. homologous position with respect to other mammalian systems
b. purified protein(s)
c. immunoblotting

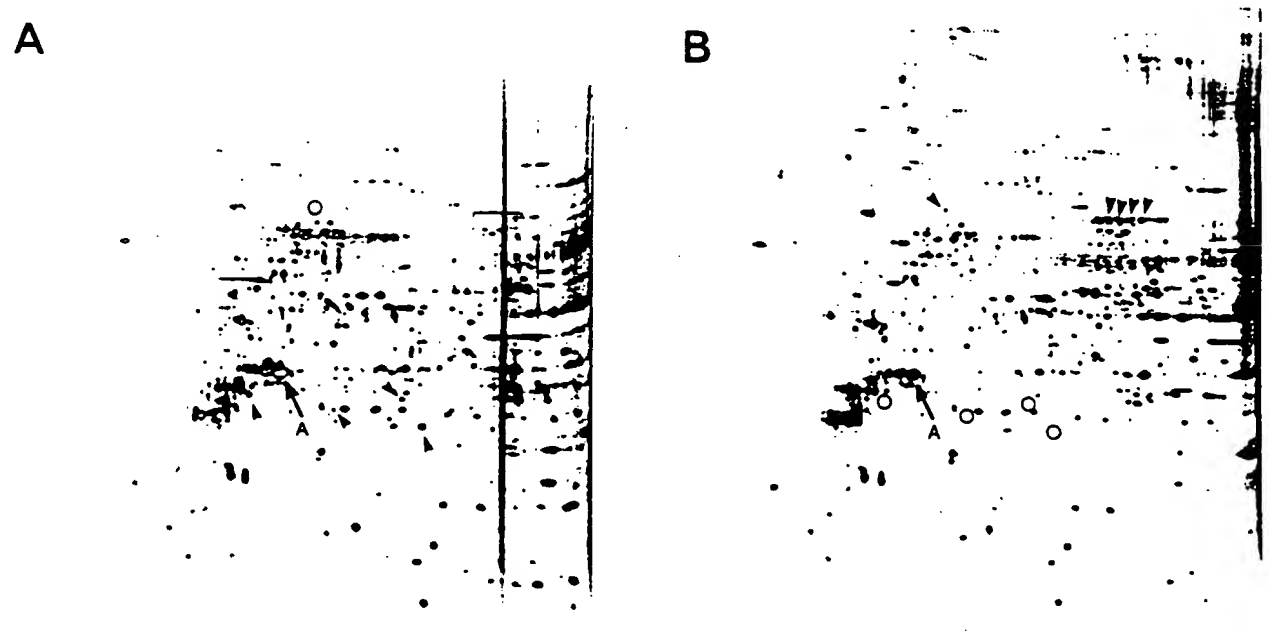

Figure 4. 2-DE analysis of a case of breast carcinoma (BC). Comparison of 2-DE quality and some differences in detected spots (arrow heads indicate increased intensity and circles or bracket indicate decreased intensity of the same spots) between (A) enzymatically and (B) nonenzymatically (scraped) tissue preparation.

3.2.2 Comparison of different methods for preparing cells from fresh tumor tissue

Samples were prepared from breast and lung carcinomas using either an enzymatic treatment with collagenase/elastase or using nonenzymatic preparations (Fig. 4). A number of differences in the protein profiles were observed in the resulting 2-DE gels, some of which are indicated in Figs. 4a and b. These differences include both increases and decreases in spot intensity. These differences may result from degradation of high molecular weight polypeptides during enzymatic treatment, increased solubilization of polypeptides, or may have other causes. For many tumors, it was only possible to obtain small amounts of material since they were reserved for other examinations. In these cases, samples could be prepared for 2-DE using either needle aspiration or scraping. Figure 5a shows a 2-DE gel prepared from squamous lung carcinoma (LS) cells collected by needle aspiration and Fig. 5b shows a gel prepared from the same tumor by scraping. In this case, a number of differences were recorded between the two procedures, some of which are arrowed in Fig. 5. Samples obtained from other tumors (breast and lung) generally showed fewer differences between these two methods of cell sampling (not shown). These data show that different nonenzymatic extraction procedures may yield different polypeptide patterns. However, the number of spots with a large

Figure 5. 2-DE analysis of a case of lung cancer (LS). Comparison of 2-DE gel quality and detected spots (arrow heads and circles) between (A) aspirated (needle aspiration) and (B) scraped preparations from fresh tissue.

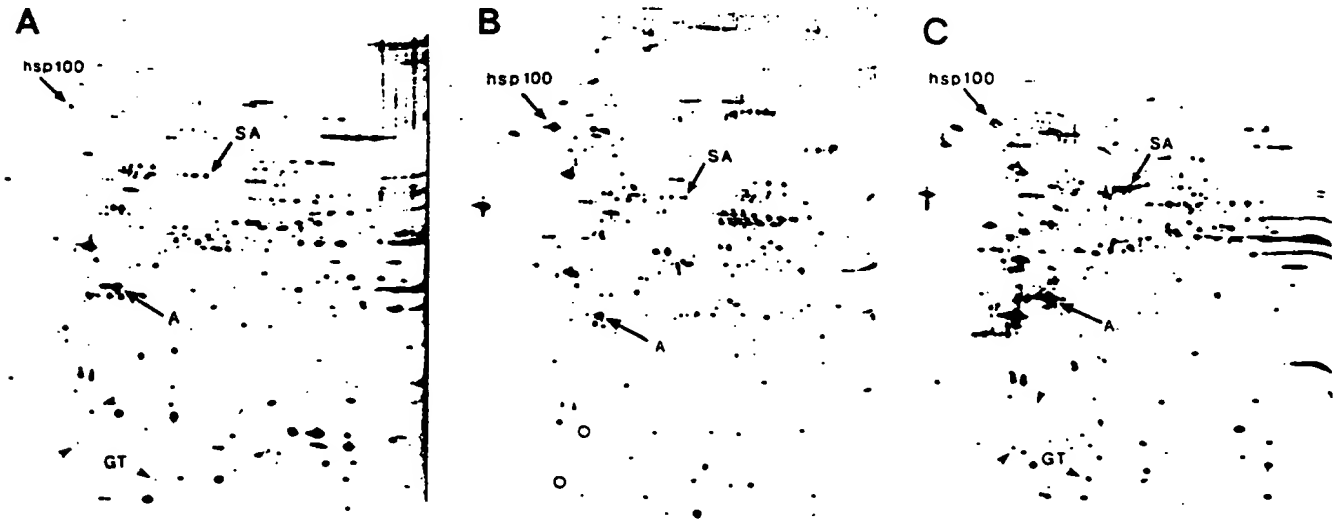

Figure 6. 2-DE analysis of three other types of tumors, (A) hypernephroma, (B) an adenoma of the thyroid and (C) corpus cancer, using the nonenzymatic preparation technique. Arrowheads and circles indicate some cytosolic polypeptides.

difference in intensity were lower than when a nonenzymatic preparation was compared with an enzymatic preparation.

2-DE maps of satisfactory quality were prepared by a third procedure. Cells were released from small pieces of tumor by squeezing (see Section 2). Some examples of this are shown in Fig. 6 where 2-DE maps derived from a case of hypernephroma, KH (Fig. 6a), a case of thyroid tumor, TA (Fig. 6b) and a case of corpus cancer, CP (Fig. 6c) can be seen. We conclude that nonenzymatic techniques are useful for 2-DE analysis of a number of different tumors. The quality of the resulting gels is comparable to that obtained using cultured cells (compare the gels in Fig. 2 with those in Fig. 4, 6 and 7). Which of these methods will be optimal will, in our experience, depend on the tumor material. For example, very small tumors are preferably extracted by squeezing; on the other hand, breast cancers (which are often fibrous) yield satisfactory samples using scraping.

3.2.3 Purification of cells on percoll gradients

We considered the possible advantage of separating viable cells from dead cells, erythrocytes, and debris using discontinuous Percoll gradients. Cells collected

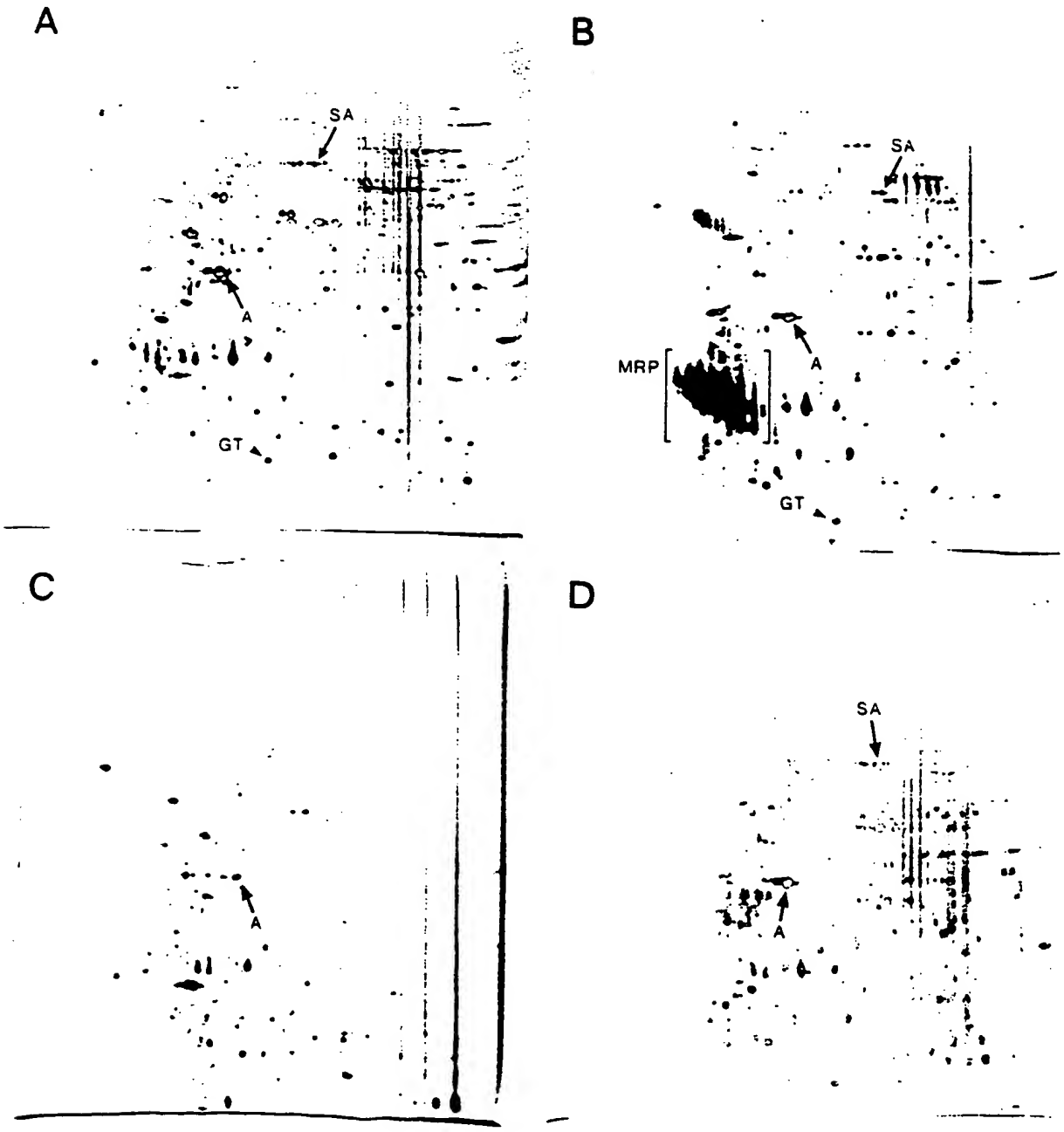

Figure 7. 2-DE analysis of polypeptides from viable (b and d) and nonviable (a and c) cells of an adenocarcinoma of the lung (LB), separated using discontinuous Percoll density gradient. Nonenzymatic preparation technique (a and b) and enzymatic preparation technique (c and d) are compared.

from the interphase showed a viability of more than 90% as judged by trypan blue exclusion test. However, it as found that the yield of viable cells decreased dramatically if the tissue resection was not immediately processed. To study the effect of lysis of cells during the preparation procedure, 2-DE maps were prepared from nonenzymatically extracted cells of case LB collected from the top fraction (nonviable, Fig. 7a) and interphase fraction (viable, Fig. 7b). These 2-DE maps were compared with corresponding fractions (nonviable, Fig. 7c, and viable, Fig. 7d) of enzymatically extracted cells. One clear disadvantage of the enzymatic technique was that when loss of cell viability occurred during preparation, a dramatic loss of high molecular weight polypeptides was observed (Fig. 7c). This was probably due to degradation of intracellular proteins. However, nonenzymatic preparations showed fewer differences between viable and nonviable cells: The most pronounced alteration was a decrease of a group of mucine related proteins (Fig. 7b). We conclude, therefore, that discontinuous Percoll gradient is necessary after enzymatic extraction of cells, but can be omitted from the nonenzymatical tumor sample preparation procedure.

We used the MDA-231 cell line to study the effects of cell lysis and leakage of cytosolic polypeptides during sample preparation. Remarkably, after 30, 50, 80 and 140 min of incubation in PBS/PIH at 0°C, no significant changes were observed in the 2-DE pattern (not shown). Although loss of cell viability may not result in protein degradation when cells are incubated in the presence of protease inhibitors, loss of cytosolic proteins would be expected during pelleting of cells. We monitored the loss of lactate dehydrogenase (LDH) activity into the supernatant during incubation in PBS of MDA-231 and MCF-7 breast cancer cells at 20°C. In both cases, loss of viability was paralleled by release of LDH from the cells (Fig. 8). After 5 h, 70% of the MCF-7 cells, but only 30% of the MDA-231 cells were dead (not shown).

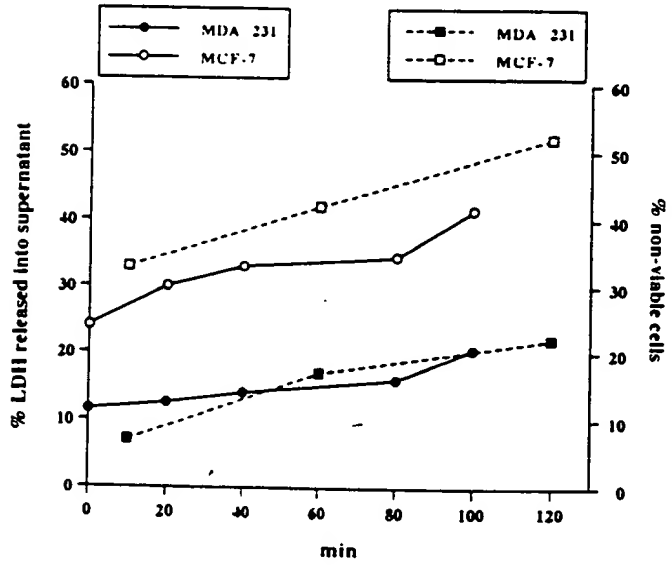

Figure 8. The relative release (fraction in supernatant of total) of lactate dehydrogenase activity (LDH) and cella viability *versus* incubation time of the mammary carcinoma cell lines MDA-231 and MCF-7 during incubation in PBS at 20°C.

These data indicate the impact of a rapid preparation procedure, at low temperature, of fresh tumor samples. Experiments have also been performed using only 1.07 g/mL Percoll (Fig. 6c and Fig. 1, left test tube) in order to remove erythrocytes. One clear advantage with this procedure, which today is routinely utilized, is a higher yield of viable cells, probably due to decreased sample preparation time.

4 Discussion

We describe procedures for sample preparation from solid tumors for 2-DE. 2-DE maps could be derived from solid tumors which were similar in quality to those obtained from cultured cells. Compared to methods using frozen material, the resolving power of the 2-DE technique is increased, allowing examination of a large number of polypeptides from tumors of different malignancies. Other investigators [12,22] have used samples from frozen tumors to derive 2-DE maps. We have previously described disadvantages encountered using frozen tumor samples including variations in contaminating proteins between different samples [3]. The methods described here are based on the preparation of cells from tumors without enzymatic digestion. The enzymatic step could be avoided since malignant cells usually grow as solid masses which are not strongly attached to the matrix. Furthermore, we found that omitting the enzymatic digestion alleviated the necessity of purifying viable tumor cells on Percoll gradients. This was in sharp contrast to enzymatically treated samples, where loss of viability leads to loss of high molecular weight proteins (Fig. 7c).

At least in the case of lung cancer, viable and nonviable cells showed small differences in respect to 2-DE maps. Presumably, protease inhibitors penetrate cells and inhibit proteolysis. In model experiments, we observed leakage of cytosolic protein (LDH) from the cells in parallel to loss of viability. Apparently, however, only a limited decrease of the level of low molecular weight cytosolic polypeptides was detected using silver staining combined with visual inspection. We have found that although some tumors are well suited for the preparation procedure described, others are not. In general, good results were obtained using tumors of the lung, breast, corpus and lymphomas. In contrast, cells from thyroid adenomas and hypernephroma showed poor viability. We were in these cases unable to separate nonviable cells from viable cells, and we can therefore not evaluate the consequence of the loss of viability on 2-DE patterns, apart from a loss of some low molecular weight cytosolic polypeptides.

Highly differentiated tumors may show lower viability as compared with poorly differentiated tumors (Dr. Farkas Vanky, personal communication). A number of samples from thyroid tumors were prepared for 2-DE but most cases showed poor viability. We believe that special care is needed during preparation of generally highly differentiated tumor groups. The difference between loss of viability/leakage of LDH of the more differentiated MCF-7 cells and the less differentiated MDA-231 cells is in line

with these observations (Fig. 8). A number of potential and interesting markers, like tropomyosin isoforms, cytokeratins and heat shock proteins, appear to be insensitive to loss of viability during the preparation procedure. We have to date made numerous observations of alterations in the expression of these polypeptides in breast cancers and lung cancers.

Another problem that may occur, irrespective of sample preparation techniques used, is admixture of lymphocytes. These cases are easily detectable in smears and it may therefore be possible to select lymphocyte specific spots as "internal markers" for the 2-D PAGE analysis. Studies using this approach are in progress. Many of the polypeptides identified are structural (Table 1). Since the expression of many of these polypeptides are known to vary between normal and malignant cells, the possibility to determine their expression simultaneously is appealing. In the specific case of breast cancer, alterations in the expression of intermediate filament proteins (cytokeratins) are known to occur during tumor progression [23]. Other proteins known to be differentially expressed between normal cells and transformed cells are tropomyosins, numatrin/B23, heat shock proteins and PCNA. To this end, we have observed alterations in the expression of cytokeratin 8, hsp 90, and non-muscle tropomyosin isoform 2 during malignant progression. (Okuzawa *et al.*, in preparation and Franzén *et al.*, in preparation).

The method of choice for sample preparation from tumor tissues will depend on the properties of the tumor material studied. It may be important to use only one method when comparing cases within one group, as differences were observed between methods. The advantages of the nonenzymatic techniques are (i) that it minimizes contamination with connective tissue, (ii) that problems with contamination of serum proteins are avoided, and (iii) that separation of viable and dead cells is not necessary. Hereby the revolving power of 2-D PAGE is maximized for the analysis of human tumors and studies on inter-tumor variations in gene expression are facilitated. In addition, the polypeptide patterns obtained may be more representative for the *in vivo* tumor cell since the use of enzymes and incubations have been minimized.

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Reference points for comparisons of two-dimensional maps of proteins from different human cell types defined in a pH scale where isoelectric points correlate with polypeptide compositions

A highly reproducible, commercial and nonlinear, wide-range immobilized pH gradient (IPG) was used to generate two-dimensional (2-D) gel maps of [^{35}S]methionine-labeled proteins from noncultured, unfractionated normal human epidermal keratinocytes. Forty one proteins, common to most human cell types and recorded in the human keratinocyte 2-D gel protein database were identified in the 2-D gel maps and their isoelectric points (p*I*) were determined using narrow-range IPGs. The latter established a pH scale that allowed comparisons between 2-D gel maps generated either with other IPGs in the first dimension or with different human protein samples. Of the 41 proteins identified, a subset of 18 was defined as suitable to evaluate the correlation between calculated and experimental p*I* values for polypeptides with known composition. The variance calculated for the discrepancies between calculated and experimental p*I* values for these proteins was 0.001 pH units. Comparison of the values by the *t*-test for dependent samples (paired test) gave a *p*-level of 0.49, indicating that there is no significant difference between the calculated and experimental p*I* values. The precision of the calculated values depended on the buffer capacity of the proteins, and on average, it improved with increased buffer capacity. As shown here, the widely available information on protein sequences cannot, *a priori*, be assumed to be sufficient for calculating p*I* values because post-translational modifications, in particular *N*-terminal blockage, pose a major problem. Of the 36 proteins analyzed in this study, 18–20 were found to be *N*-terminally blocked and of these only 6 were indicated as such in databases. The probability of *N*-terminal blockage depended on the nature of the *N*-terminal group. Twenty six of the proteins had either M, S or A as *N*-terminal amino acids and of these 17–19 were blocked. Only 1 in 10 proteins containing other *N*-terminal groups were blocked.

1 Introduction

As compared with carrier ampholyte isoelectric focusing (CA-IEF), the application of immobilized pH gradients (IPGs) in the first dimension in 2-D gel electrophoresis offers improved reproducibility [1] because the nature of the pH gradient makes the resulting focusing positions insensitive to the focusing time [2] and to the type of sample applied [3]. The recently introduced ready-made IPG strips [4] seem to be an ideal substitute for the carrier ampholyte gradients, which until now have been the most commonly used first dimensions in 2-D gel electrophoresis. The availability of standardized first dimensions opens the possibility of comparing 2-D gel maps of various cell types generated in different laboratories, provided that the focusing positions of a number of easily recognizable polypeptide spots common to the cell types in question are known. Even though this approach is limited to experiments performed with the same standardized IPG, the flexibility provided by IPGs allows the pH gradient to be adjusted to the requirements of a particular experiment.

Exchange and communication of 2-D gel protein data requires a pH scale that is independent of the particular IPG used and by which the results can be described. The introduction of carbamylation trains and the relation of focusing positions to the spots in these trains represented a step forward towards solving the reproducibility problem experienced with carrier ampholyte focusing [5]. Problems associated with the use of carbamylation trains were mainly due to lack of temperature control and to the use of nonequilibrium focusing conditions. Accordingly, the pattern variation involved not only the resulting pH gradients, but also the relative spot positions as related to each other and to spots in the carbamylation trains. Even though the question of reproducibility has, to a large extent, been solved, the carbamylation trains are still not ideal as markers because the spots in the trains do not represent defined entities but rather a large number of differently carbamylated peptides having close p*I* values. As a result, the spots are large and poorly defined as compared to the ordinary polypeptide spots in 2-D gel maps.

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Abbreviations: **CA-IEF**, carrier ampholyte-isoelectric focusing; **SSP**, sample spot number

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Neidhardt *et al.* [6] defined the pH gradient in 2-D gel experiments by p*I* markers whose p*I* values were calculated from the amino acid composition. Focusing positions of other polypeptides could be predicted from their composition but the p*K* values needed for the p*I* calculations were unknown. Various groups employing this approach do not use the same pK values [6, 7] and therefore, the p*I* values derived in this way cannot be expected to describe the variation of the hydrogen ion activity. In spite of this fact, it is still possible to make approximate predictions of focusing positions because the p*K* values used to define the pH gradient are also used to calculate p*I* values and to predict the focusing positions. Errors in p*K* assignments are therefore compensated. A pH scale which correctly reflects the variation in hydrogen ion activity during focusing should improve the precision of the predictions, but this has never been implemented with CA-IEF focusing as a first dimension in 2-D gel electrophoresis. The main reason for this are the problems associated with pH measurements in focused gels containing high concentrations of urea.

IPGs can be described from the concentration variation of the immobilized groups, provided that the p*K* values of these groups are known for the conditions prevailing during focusing. To avoid measurements on gels, Gianazza *et al.* [8] suggested the use of p*K* values derived by addition of determined p*K* shifts. Recently, direct determinations of p*K* differences between immobilized groups in IPGs were made by determining p*I*-p*K* values in overlapping narrow-range IPGs [9, 10] and the results verified the applicability of the Gianazza approach. A description of the focusing results in a pH scale, which correctly describes the variation of the hydrogen ion activity for the focusing conditions used, not only allows the comparison of 2-D gel maps generated with different IPGs, but also opens the possibility for correlating the focusing position of a polypeptide with its composition [9]. Experiments by Bjellqvist *et al.* [9, 10] have implied that pH scales showing good correlation between calculated and experimental p*I* values can be derived for any of the conditions commonly used for focusing in connection with 2-D gel electrophoresis. These pH scales are then defined through the p*K* values of the immobilized groups in the IPG containing gel. To be useful for interlaboratory comparisons, however, the pH scale has to be defined through p*I* values of easily recognizable spots present in the 2-D gel map. So far, p*I* determinations in a useful pH scale, combined with determinations of pK values needed for p*I* calculations, have only been made for the pH range 4.5–6.5 at 10°C [9]. CA-IEF focusing as described by O'Farrell [11] does not control the temperature of the first dimension, which can be expected to be slightly above room temperature. With IPGs, the temperature commonly used is about 20°C [4, 12] or 25°C [13] and this is a critical parameter that needs to be controlled [14].

The present work was designed to compare 2-D gel maps of different cell types in a laboratory applying both CA-IEF and IPG focusing at a common temperature. To this end we have generated 2-D gel maps of proteins from noncultured, unfractionated normal human epidermal keratinocytes with IPG in the first dimension and a focusing temperature of 25°C. We have used commercial nonlinear, wide-range IPG strips which give 2-D gel maps that are closely similar to the ones resulting with the CA-IEF technique used to establish the human keratinocyte database [15]. As an initial step towards interlaboratory comparisons of results obtained with the nonlinear gradient as a first dimension we report here on the focusing positions of 41 known proteins that are common to most human cell types. The pH range covered corresponds to the range in classical CA-IEF 2-D gel electrophoresis and in order to use these proteins as internal standards for comparing 2-D gel maps generated with other IPGs we determined their p*I* values with narrow-range IPGs in the first dimension. We have compared the calculated *versus* experimental p*I* values and show that it is necessary to have further information (absence or presence and nature of posttranslational modifications), in addition to amino acid composition to be able to calculate p*I* values that correspond to the actual experimental values. The p*K* values used for the calculations are provided and the usefulness of p*I* prediction in relation to database information is discussed. Furthermore, we comment on the possibility of using experimentally determined p*I* values to verify the available database information on polypeptide composition.

2 Materials and methods

2.1 Apparatus and chemicals

Equipment for isoelectric focusing and horizontal SDS electrophoresis (Multiphor® II electrophoresis chamber, Immobiline® strip tray, Multidrive XL programmable power supply, Macrodrive power supply and Multitemp® II) was from Pharmacia LKB Biotechnology AB (Uppsala, Sweden). Vertical second-dimensional gels were run in the home-made equipment described in [15]. The IPG strips with the wide-range nonlinear pH gradient were either Immobiline DryStrip® pH 3–10 NL, 180 mm or alternatively 160 mm long IPG strips with a corresponding pH gradient. In both cases the IPG strips were delivered by Pharmacia LKB. Immobiline, Pharmalyte, Ampholine, GelBond as well as PAG film and the ready-made horizontal SDS gels (ExcelGel® XL SDS 12–14) were also from Pharmacia LKB. Purified proteins and peptides were from Sigma (St. Louis, MO).

2.2 Sample preparation

Preparation and labeling of unfractionated keratinocytes as well as fibroblasts have been described in [16]. Cells were lysed in a solution containing 9.8 M urea, 2% w/v NP-40, 100 mM DTT and 2% v/v Ampholine pH 7–9.

2.3 2-D gel electrophoresis

First-dimensional focusing was performed according to Görg *et al.* [2] with some minor modifications, as described in [9]. Rehydration of the IPG strips was made in a solution containing 9.8 M urea, 2% w/v CHAPS, 10 mM DTT and 2% v/v carrier ampholyte mixture. The carrier ampholyte mixture consisted of 2 parts Pharmalyte

4–6.5, 1 part Ampholine pH 6–8 and 1 part Pharmalyte pH 8–10.5. Usually, cathodic sample application was used and the samples were diluted 2–20 times in a solution containing 9.8 M urea, 4% w/v CHAPS, 1% w/v DTT and 35 mM Tris base. For acidic application, the Tris-base was substituted with 100 mM acetic acid. The degree of dilution and sample volume (20–100 µL) depended on the particular sample and the IPG, and whether visualization of the proteins was to be done by Coomassie Brilliant Blue or silver staining. With the wide-range non-linear IPG, 10–30 µg of total protein was loaded for silver staining and 100–200 µg for Coomassie staining. Focusing was done overnight with Vh products in the range of 45–60 kVh with 160 mm long strips and 50–70 kVh with 180 mm long strips. Solubilization of polypeptides and blocking of -SH groups prior to the second-dimensional run, as well as loading on the second-dimensional gel was done as described in [9]. The stacking gel was omitted and 5–10 mm were left at the top of the second-dimensional gel for applying the IPG strip. The space was filled with electrode buffer containing 0.5% w/v agarose. Casting, running, staining and autoradiography were carried out as described in [15].

2.4 Experimental determination of p*I* values

The determination of the p*K* differences between Immobilines p*K* 4.6, p*K* 6.2 and p*K* 7.0 necessary for the calibration of the pH scale at 25°C in 9.8 M urea was done as described in [9] with the same narrow-range IPGs. The pH scale was defined by setting the p*K* value of Immobiline p*K* 4.6 equal to 4.61 [9] and the determined p*K* differences gave the p*K* values of Immobilines p*K* 6.2 and p*K* 7.0, equal to 5.73 and 6.54, respectively. The p*K* differences found are in good agreement with values derived from [17] and [8] by extrapolation to 9.8 M urea concentration. As in [9], additional narrow-range recipes have been used for determining p*I* values. With narrow-range IPGs extending to pH values higher than the p*K* value of Immobiline p*K* 7.0, anodic sample application was used with acetic acid added to the sample solution. Otherwise, cathodic sample application was used with the same sample buffer as for wide-range IPGs.

2.5 Protein compositions used for p*I* calculations

With the exception of vimentin, protein compositions are from the Swiss-Prot database [18]. For vimentin, we used the data from [19], where the amino acid at position 41 is a D instead of a S. Information in the Swiss-Prot database on phosphorylation has been disregarded because it was known from earlier studies (J. E. Celis, unpublished results) that the spots in question corresponded to the unphosphorylated forms of the peptides.

2.6 Calculation of p*I* values

For the p*I* calculations it was assumed that the same p*K* value could be used for an amino acid residue in all polypeptides and in all positions in the peptide except for *N*- or *C*-terminally placed amino acids. For the p*K* values of the *N*-terminal amino groups the effect of the different substituents on the α-carbon were taken into account. The calculations of p*I* values were made with the aid of the IPG-maker program [20].

2.7 p*K* values used for p*I* calculations

For the carboxyl terminal group and internal glutamyl and aspartyl residues the same p*K* values were used as in [9]. For *C*-terminal glutamyl and aspartyl residues, separate p*K* values were derived with the aid of the Taft equations [9, 21]. The p*K* values of histidyl groups were calculated from the p*I* values of human carbonic anhydrase I as in [9]. For *N*-terminal glycine a p*K* value of 7.50 was used. The p*K* shift caused by a substituent on the α-carbon was assumed to be identical with the p*K* shift the substituent caused for the amino group in the amino acid, *i.e.* 2.28 pH units were subtracted from the p*K* values for the amino groups in the amino acids given in [22, 23]. The approximate p*K* value of 9 for the cystenyl group was taken from [24]. For tyrosyl and arginyl groups we used the p*K* values for the amino acids [22, 23]. For lysyl groups the effect of high urea concentration on amino groups was taken into account and 0.5 pH units were subtracted from the amino acid p*K* value. These last three p*K* values are far from the pH range under study and the results found would have been the same if lysyl and arginyl groups were assumed to be fully ionized while the ionization of tyrosyl groups were neglected. A complete list of the p*K* values used is given in Table 1.

Table 1. p*K* Values used for the ionizable groups in peptides 9.8 M urea, 25°C

Ionizable group	p*K*
C-terminal	3.55
N-terminal	
Ala	7.59
Met	7.00
Ser	6.93
Pro	8.36
Thr	6.82
Val	7.44
Glu	7.70
Internal	
Asp	4.05
Glu	4.45
His	5.98
Cys	9
Tyr	10
Lys	10
Arg	12
C-terminal side chain groups	
Asp	4.55
Glu	4.75

2.8 Statistical analysis

Statistical comparisons of the experimental and calculated p*I* values were done on an Apple Macintosh IIsi using the statistical package Statistica/Mac, release 3.0b (from StatSoft Inc., Tulsa, Oklahoma). Calculated and experimental p*I* values were compared by the *t*-test for

correlated samples (paired *t*-test). The normality of p*I* differences was estimated graphically by probability plots. The variances of the data presented here and the similar data on plasma and liver proteins in [9] were compared by the F-test.

3 Results and discussion

3.1 Identification of polypeptides and p*I* determinations

The 2-D gel maps of [^{35}S]methionine-labeled proteins from noncultured, unfractionated normal human keratinocytes, focused with the nonlinear, wide-range IPG and CA-IEF pH gradients in the first dimension, are shown in Figs. 1 and 2, respectively. The IPG extends to higher pH values but otherwise the two patterns are very similar and most of the spots in the IPG pattern can be directly related to the corresponding spots in the CA-IEF gel. To obtain comparable patterns it was important to keep the focusing temperature as similar as possible. Compared to other studies [1–4, 9, 10, 12–14], we increased the urea concentration in the focusing gel to 9.8 M because keratins streaked badly in the focusing dimension when 8 M urea was used, presumably due to

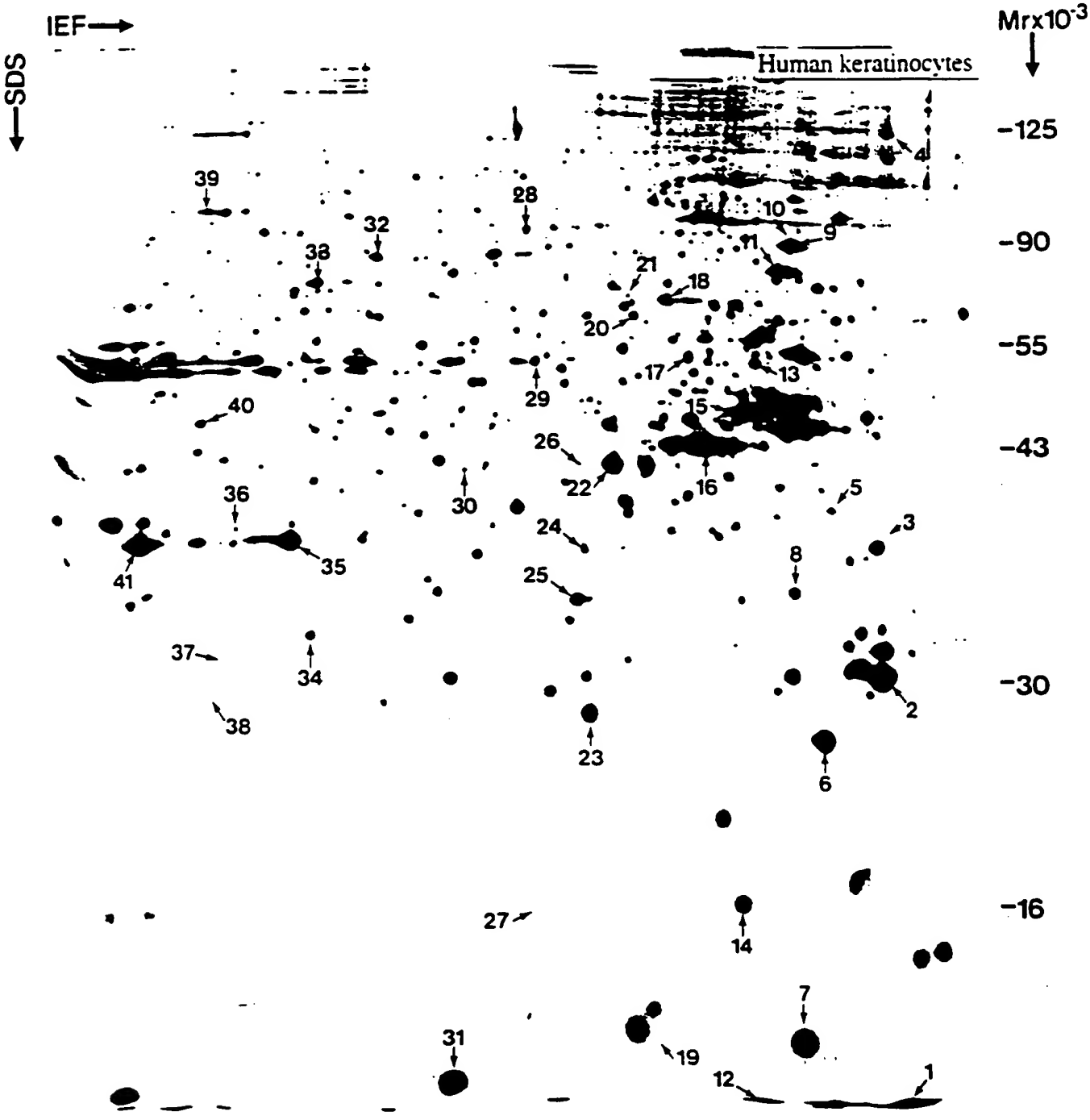


Figure 1. 2-D gel protein map of [^{35}S]methionine-labeled proteins from noncultured, unfractionated normal human keratinocytes focused with the nonlinear, wide-range IPG in the first dimension. The position of the 41 proteins analyzed in this study is indicated.

aggregates of acidic and basic keratins. An increase in urea concentration to 9 M or more eliminated these streaks; apart from this effect, no other major changes in the focusing positions were observed. In Fig. 1 we have indicated the positions of 41 known proteins from the human keratinocyte 2-D gel database that are most likely common to most human cell types. The choice was made because these proteins are easy to identify with certainty. With the exception of stratifin (spot 2), involucrin (spot 4) and keratin 14 (spot 15), which are all epithelial markers, these proteins are also present in human fibroblasts (Fig. 3) and lymphocytes (results not shown), and therefore can be used as landmarks for comparing 2-D gel maps derived from different cell types. In Table 2 the 41 proteins are listed together with their sample spot numbers (SSP) in the human keratinocyte protein database and p*I* values determined in 2-D gel maps generated with narrow-range IPGs in the first dimension.

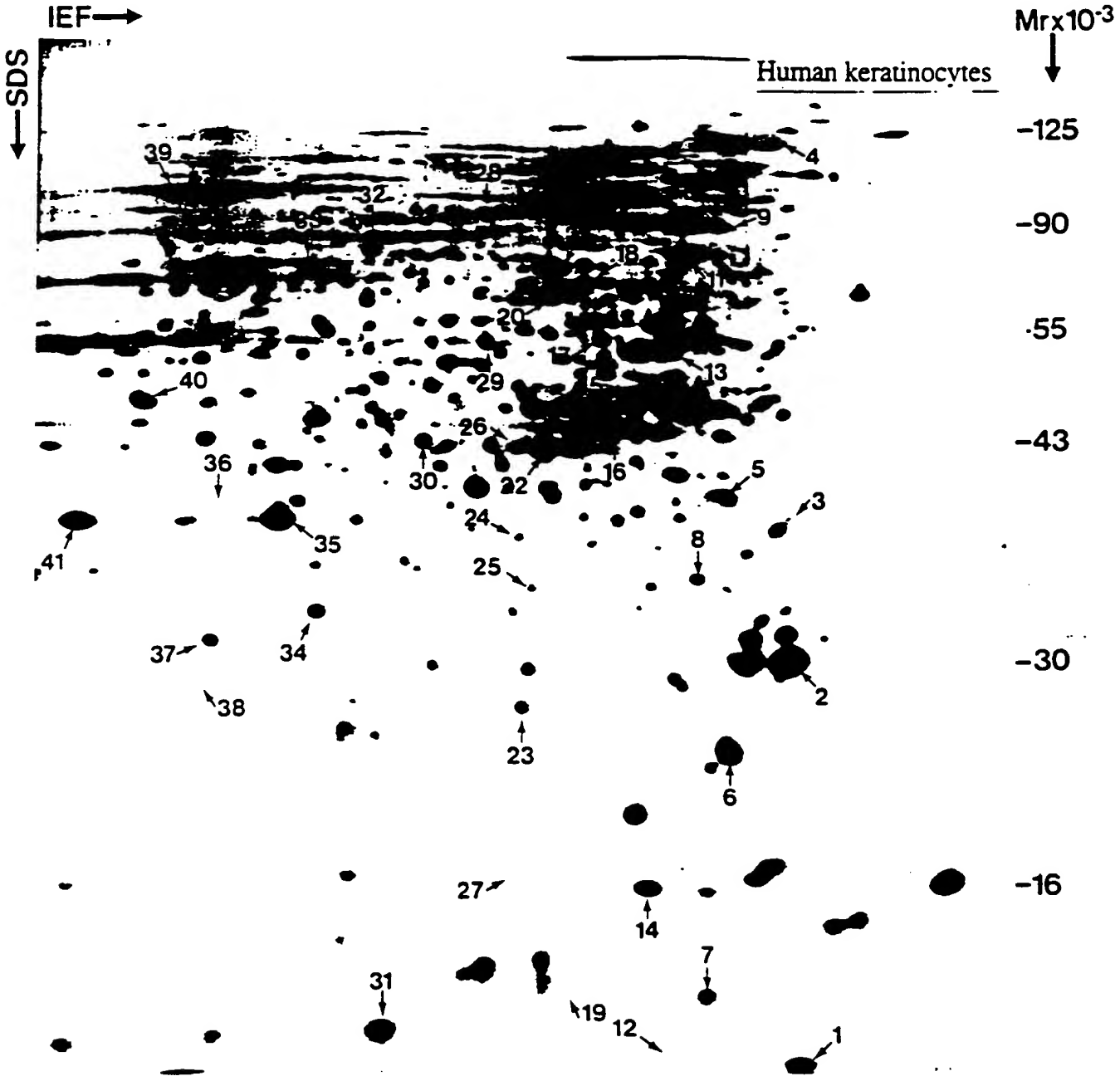


Figure 2. 2-D gel protein map of [^{35}S]methionine-labeled proteins from noncultured, unfractionated normal human keratinocytes focused with CA-IEF in the first dimension. The position of the 41 proteins analyzed in this study is indicated.

Table 2. Proteins from the human keratinocyte database localized in 2-D gels run with IPGs as first dimension

Number in Figs. 1–3	Protein name	IEF SSP number[a]	Experimental *pI* value	Calculated *pI* value	Discrepancy (pH units)	Calculated net charge at experimental *pI* value	Buffer capacity charge units pro pH unit	*N*-terminal	Recalculated for suspected blockage		*N*-terminal	Swiss-Prot accession number
									pI value	Discrepancy pH units	Net charge	
1	CaN 19	9027	4.46	..	..	..	..					
2	Stratilin, bovine 14-3-3 related protein	9109	4.58	..	..	..	..					
3	Proliferating nuclear antigen (PCNA)/cyclin	9226	4.58	4.57	-0.01	-0.1	20.8	M				P12004
4	Involucrin[c]	9703	4.63	4.63	0.00	-0.3	70.1	M				P07476
5	Nucleolar protein B23	8207	4.75	4.64	-0.11	-3.2	30.4	M				P06748
6	Translationally controlled tumor protein	8114	4.79	4.84	0.05	0.6	13.1	M[b]				P13693
7	Thioredoxin	8006	4.86	4.82	-0.04	-0.3	7.1	V[b]				P10599
8	Annexin V	8213	4.89	4.88	-0.01	-0.1	20.3	A[c]				P08758
9	Heat shock protein 90-β	8611	4.95	4.94	-0.01	-0.5	56.2	P				P07900
10	Heat shock protein 90-α	2629	4.97	4.97	0.00	0.2	53.6	P				P08238
11	Glucose regulated protein 78 (BiP)	8515	4.99	4.98	-0.01	-0.6	37.5	E				P11021
12	Calcyclin	8017	5.02	5.32	0.30	1.3	3.6	M	5.09	0.07	0.3	P06703
13	Vimentin	8417	5.05	5.06	0.01	0.2	27.1	S				P08670
14	Initiation factor 4D	8016	5.05	5.08	0.03	0.2	7.6	A[c]				P10159
15	Keratin 14	7305	5.08	5.09	0.01	0.2	21.0	T				P02533
16	β-Actin	7316	5.21	5.21	0.00	0.06	13.3	D[c]				P02570
17	Heat shock protein 60	6403	5.23	5.24	0.01	0.1	17.5	A[b]				P10809
18	Heat shock cognate 71kD	6501	5.28	5.37	0.09	1.8	18.1	M	5.32	0.04	0.8	P11142
19	Cystatin	6011	5.30	5.38	0.08	0.2	3.0	M				P01040
20	Epiplastin	6112	5.34	5.41	0.07	1.1	17.7	M	5.36	0.02	0.3	P13797
21	Calelectrin	5628	5.35	5.37	0.02	0.5	23.3	A[c]				P08133
22	Plasminogen activator inhibitor-2	6314	5.38	5.46	0.08	0.9	10.7	M	5.37	0.01	0.07	P05120
23	Glutathione S-transferase π	5101	5.43	5.44	0.01	0.08	3.9	P				P09211
24	Annexin VIII	5213	5.45	5.56	0.11	1.0	8.7	M	5.46	0.01	0.05	P13928
25	Annexin III	5204	5.46	5.63	0.17	1.4	8.4	M	5.52	0.06	0.5	P12429
26	Adenosine deaminase	5305	5.47	5.63	0.16	1.8	10.8	M	5.54	0.07	0.8	P00813
27	Stathmin	5001	5.55	5.61	0.06	0.4	6.6	A[c]				P16949
28	Gelsolin, cytoplasmic	5608	5.59	5.58	-0.01	-0.1	16.5	V				P06396
29	Rat phosphoinoside specific protein homolog	5410	5.62		..		..					
30	Elastase inhibitor	4314	5.74	..	..	..						
31	S100, calgizarin	4006	5.75	..	..	..	..					
32	Cytvillin, ezrin	3504	5.99	5.95	-0.04	-0.5	13.2	P				P15311
33	Moesin	3515	6.11	6.09	-0.02	-0.2	9.8	P				P26038
34	Purine nucleoside phosphorylase	2108	6.11	6.45	0.34	1.8	4.4	M	6.28	0.17	0.9	P00491
35	Annexin I	2216	6.18	6.64[c]	0.46	1.6	2.5	A	6.33	0.15	0.6	P04083
36	Aldose reductase	1202	6.40	6.55	0.15	0.7	4.2	A	6.36	0.04	0.2	P15121
37	Phosphoglycerate mutase (B form)	1107	6.46	6.75	0.29	0.9	2.6	A	6.46	0.00	0.0	P18669
38	Triosephosphate isomerase	1111	6.53	6.51	-0.02	-0.04	2.3	A[b]				P00938
39	Elongation factor 2	1610	6.43	6.38	-0.05	-0.5	9.8	M				P13639
40	α-Enolase	1325	6.62	6.99	0.37	1.0	2.2	S	6.75	0.13	0.3	P06733
41	Annexin II	210	7.30	7.36	0.06	0.05	0.9	S[c]				P07355

a) SSP number in the keratinocyte database [15]
b) Peptides *N*-terminally sequenced as liver proteins [3]
c) Peptides given as *N*-terminally blocked in Swiss-Prot database

3.2 Comparison between the determined and calculated p*I* values for human keratinocyte proteins

Thirty six of the 41 proteins listed in Table 2 are found in the Swiss-Prot database. Contrary to the plasma and liver proteins used in [9], the p*I* calcuations on the proteins used in this study posed some problems that reflected the way in which they were characterized. The proteins used by Bjellqvist *et al.* [9] were either very abundant and well-characterized plasma proteins or they were identified by *N*-terminal sequencing and, therefore, the nature of the *N*-terminals (acetylated or non-acetylated) was in both cases known. The proteins used in this study have all been characterized by internal sequencing [7] and it is known that *N*-terminal acetylation occurs with high frequency in eukaryotes.

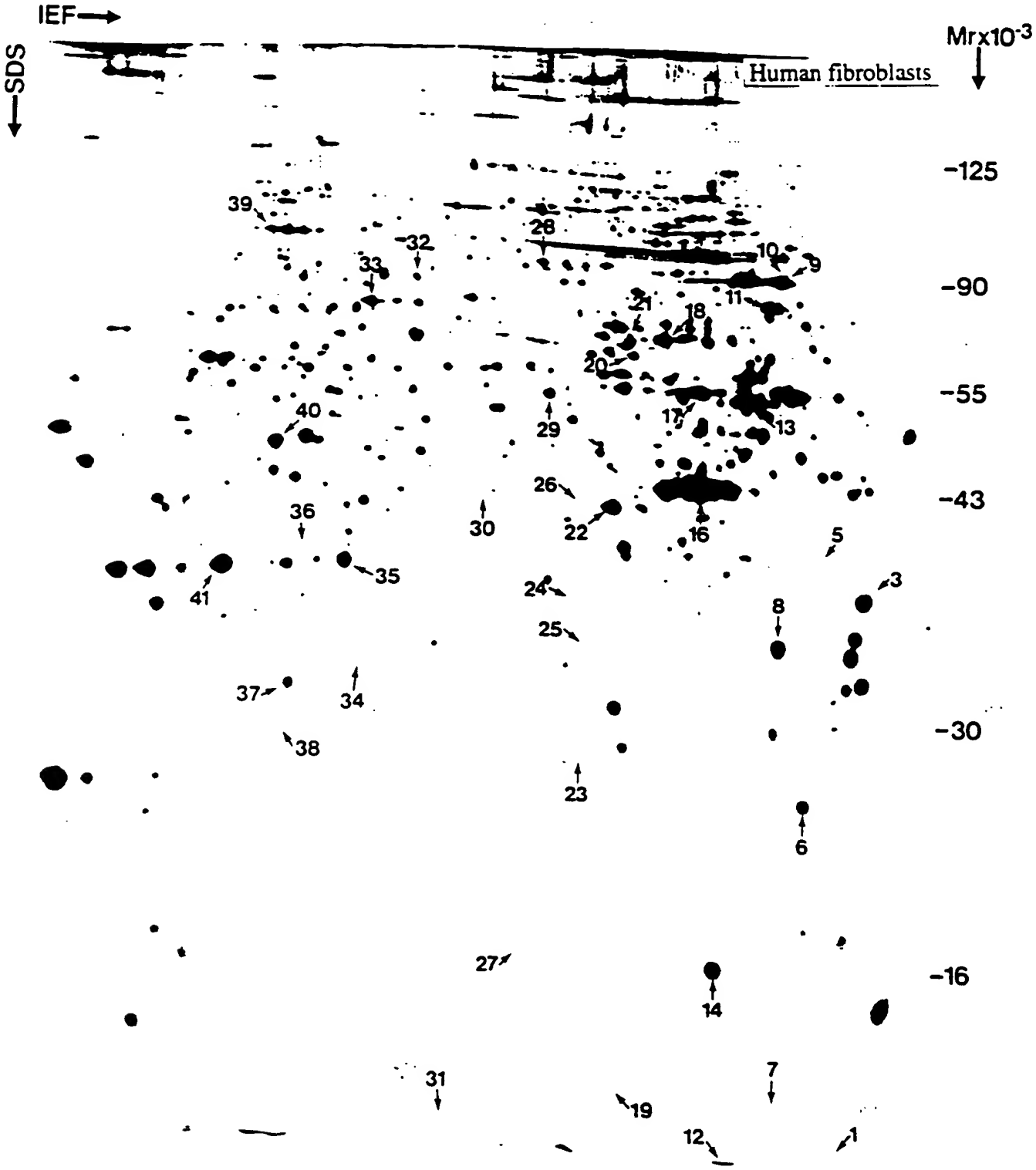


Figure 3. 2-D protein map of [^{35}S]methionine-labeled proteins from normal human fibroblasts focused with the nonlinear, wide-range IPG in the first dimension. The position of the 41 proteins analyzed in this study is indicated.

According to Brown and Robert [25], proteins with acetylated *N*-terminals correspond in weight to approximately 80% of the soluble protein in ascites cells. Based on results from *N*-terminal sequencing, at least 40% of the spots in the human liver protein 2-D gel map appear to be blocked [3]. The corresponding number, derived from 107 spots in the 2-D gel map of human T-lymphocyte proteins, falls between 60 and 65% (J. Strahler, personal communication). Information concerning *N*-terminal blockage is not normally available, and in the Swiss-Prot database only 6 of the 36 keratinocyte proteins are specified as *N*-terminally blocked. We have, within the present material, defined 18 proteins for which the *N*-terminals are very likely to be correctly described. Six of these proteins are listed in the Swiss-Prot database as *N*-terminally blocked, four represent proteins which appear in the human liver 2-D gel map and have been *N*-terminally sequenced as liver proteins [3] and the remaining eight have *N*-terminal groups other than M, S and A, *i.e.* *N*-terminals for which *N*-acetylation is uncommon [26]. In Figs. 4A, B, C and D p*I* values calculated from Swiss Prot database information are plotted against the experimentally determined p*I* values for all the keratinocyte proteins listed in Table 2 and for the 18 selected proteins, as well as for the plasma and liver proteins (data from [9] valid for 10°C)*.

The calculations show that without knowledge of the status of the *N*-terminal group, precise predictions of p*I* values for eukaryotic proteins cannot be achieved based on the information available in Swiss-Prot and similar databases. However, for proteins where the *N*-terminal status is known, we find good correlation between predicted and experimental p*I* values. When the variance of the p*I* discrepancies and the variance of calculated charges at the experimental p*I* values derived from the present data set are compared with the corresponding

* There are four plots: (A) the 36 polypeptides from normal human keratinocytes (no corrections), (B) the 36 polypeptides from Fig. 4A where p*I* values have been recalculated for 12 polypeptides with M, S and A as *N*-terminally assumed blocked, based on calculated charge, (C) the 18 selected polypeptides with information on the *N*-terminal configuration, and (D) plasma and liver proteins.

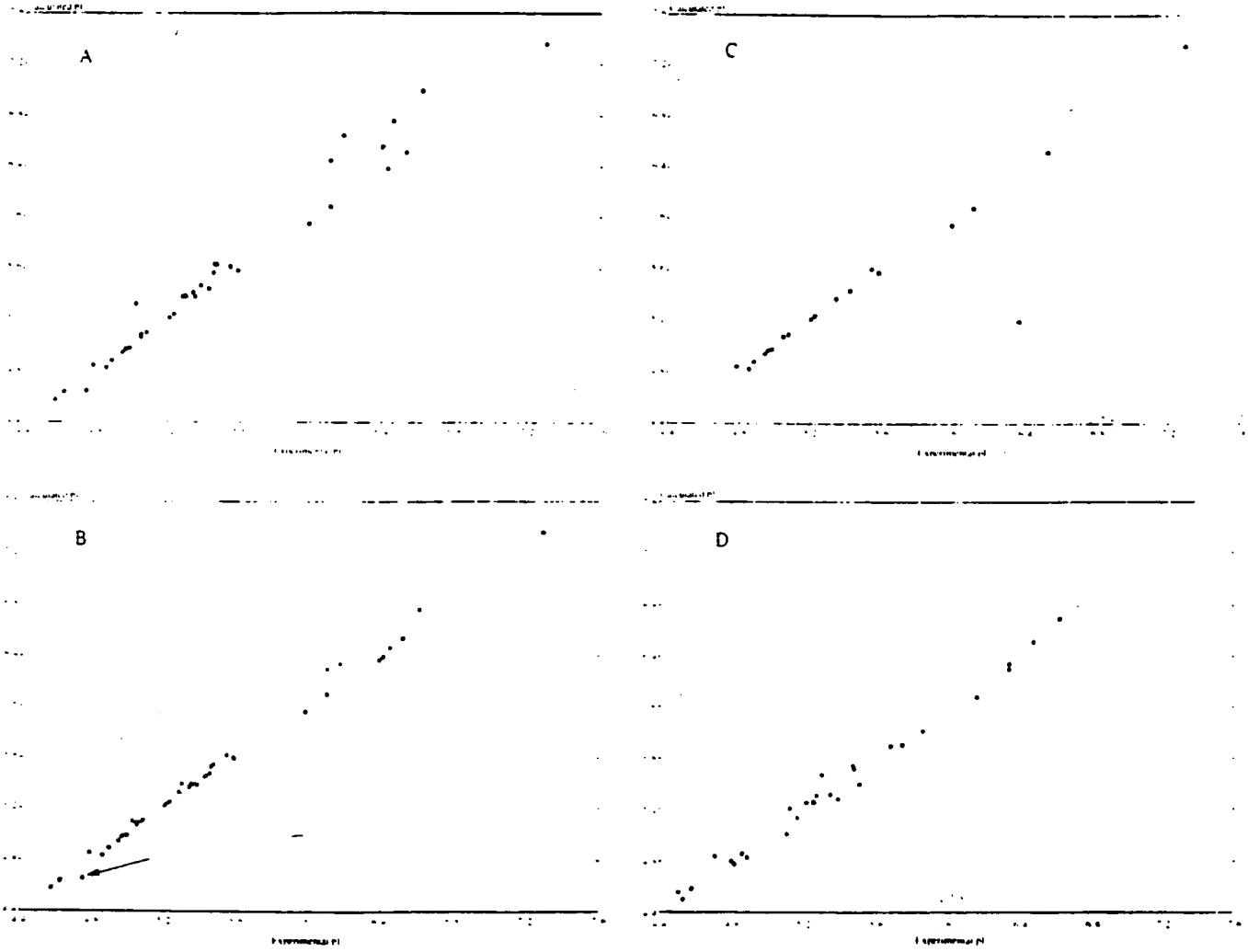


Figure 4. Calculated *vs.* experimental p*I* values. Lines are fitted using the least squares' criterion. (A) 36 polypeptides from normal human keratinocytes (no corrections). (B) 36 polypeptides from Fig. 4A (including the 18 marker polypeptides) where p*I* values have been recalculated assuming *N*-terminal blockage; x indicates recalculated p*I* values; nucleolar protein B23 is indicated with an arrow. (C) 18 polypeptides with information on *N*-terminal configuration and (D) plasma and liver proteins.

values derived from the data on plasma and liver proteins in [9] (Table 3), the present data are found to result in larger variances for the values of both p*I* discrepancies and calculated charge at the experimental p*I* value when no information on posttranslational modification is taken into consideration. Correction for possible *N*-acetylation of 12 polypeptides with M, S and A as *N*-terminal results in a smaller variance of p*I* discrepancies, although not significantly different from values derived from [9], whereas the variance of the calculated charge at the experimental p*I* value is significantly higher. For the 18 selected proteins the variance for the p*I* discrepancies is significantly smaller than for the data in [9]; however, the corresponding value for calculated charge at the experimental p*I* value does not improve to the same extent. This, we believe, reflects another difference between the two sets of proteins used for the calculations. Based on spot distributions in 2-D gel maps, the set of proteins used here has a molecular weight distribution that is more representative of the patterns observed in mammalian cells. In the study by Bjellqvist *et al.* [9] most of the high molecular weight plasma proteins had to be excluded due to their unknown content of sialic acid which made the proteins analyzed in this study heavily biased towards low molecular weight proteins. The buffer capacity of proteins normally increases with the protein's molecular weight, and the average buffer capacity of the presently selected proteins with assumed known *N*-terminals is 18 charge units/pH unit, while the corresponding value for the proteins used in [9] is only 9 charge units/pH unit. High buffer capacity can be expected to improve the agreement between calculated and experimental p*I* values. Inspection of the data presented in Table 2 for the polypeptides with assumed known *N*-terminals verifies the importance of the buffer capacity. For 8 polypeptides having buffer capacities higher than 15 charge units/pH unit, the calculations in all cases yielded p*I* discrepancies with absolute values of less than 0.02 pH units. The largest discrepancy, 0.06 pH units, was observed for annexin II and stathmin, proteins which have low buffer capacity: 0.9 and 6.6 charge units/pH unit, respectively. The probability that the focusing position of a protein with known composition will fall within a certain distance from the calculated p*I* value therefore cannot be predicted by the variance alone. The buffer capacity of the specific protein must be taken into consideration as well. As indicated by the decrease of the variance of calculated charges at the experimental p*I* value for the selected proteins, the observed improvement can not solely be due to the higher buffer capacity of the keratinocyte proteins. The two studies relate to different experimental conditions. Good agreement between experimental and calculated p*I* values implies that the proteins are defolded and a factor that may contribute to the observed improvement is a more complete defolding of proteins caused by the higher temperature and urea concentration used in this study.

The data indicated that the precision with which p*I* values can be predicted for polypeptides with high buffer capacity is better than the precision with which experimental p*I* values can be determined. If the pH is defined through the p*K* values of the immobilized groups in the IPG containing gel, the precision of the experimentally calculated data will depend on the pH difference between the p*I* and the p*K* value of the immobilized group with the closest p*K*. For the present study this will give p*I* determinations with a precision varying in the range of $\pm$ 0.02–0.05 pH units [9]. The good agreement observed between the calculated and experimental p*I* values is due to the fact that errors are mainly systematic and, as discussed in [9], they will largely be cancelled out in the calculations. A pH scale defined through the presently determined p*I* values will not necessarily reflect the variation of the hydrogen ion activity during the focusing step in an optimal way, but it still allows precise predictions of focusing positions for polypeptides with known compositions, including information on posttranslational modifications. Calculated net charge at the experimentally found isoelectric point defined in this scale will serve as a tool to verify that the polypeptide

Table 3. Mean values and variances for the difference (experimental p*I*-calculated p*I*) in pH units and calculated charges at the experimental p*I* values, respectively

	Plasma and liver proteins (8 M urea, 10°C)		Keratinocyte proteins (9.8 M urea, 25°C)					
			All peptides		All peptides after correction for *N*-acetylation		Known *N*-terminal configuration (or very likely configuration)	
Number of proteins	29		36		36		18	
	Mean	Variance	Mean	Variance	Mean	Variance	Mean	Variance
Experimental p*I*–calculated p*I*	−0.011	0.005	0.072	0.017	0.019	0.003	0.005	0.001
F-value (p*I* discrepancy)[a]	1		3.4		1.67		5	
P-level (p*I* discrepancy)[b]	0.5		0.0005		0.0721		0.0004	
Calculated charge at the experimental p*I* value	−0.070	0.227	0.321	0.871	0.009	0.444	−0.014	0.109
F-value (calculated charge at the experimental p*I* value)[a]	1		3.8		1.96		2.08	
P-level (calculated charge at the experimental p*I* value)[b]	0.5		0.0002		0.0338		0.0536	

[a] Comparison to the data in [9]. $F = S_1^2/S_2^2$, where S_1^2 is the larger of the two variances

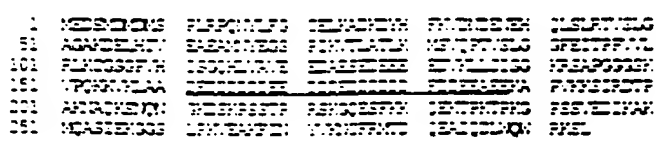
[b] $P(F(\nu_1, \nu_2) \geq F\text{-value})$, where ν_1 and ν_2 are the degrees of freedom for s_1 and s_2, respectively

composition used in the calculation is correct and complete. Exceptions to this are proteins such as involucrin and heat shock protein 90 that have very high buffer capacities. Introduction of an extra charge unit into these proteins will only result in p*I* shifts falling in the range of 0.01–0.02 pH units and the effect is that the quality of the pH definition – the precision by which p*K* values used in the calculations are given and the precision of experimental p*I* values in these cases – will limit the possibilities to verify polypeptide compostion based on the experimental p*I* value.

Statistical comparison of experimental and calculated p*I* values was done using the *t*-test for dependent samples and normality of the discrepancies was estimated by probability plots. For the 36 proteins, the *p*-level is 0.0021, indicating that a result like this is unlikely to be a chance effect and must be assumed to represent a real difference. After correction for the most likely *N*-terminal configuration, the *p*-level is 0.043 and cannot be accepted as representing the same population since the *p*-level is less than 0.05 – the traditional *p*-limit of statistical significance. For the 18 proteins with a known or very likely *N*-terminal configuration the *t*-test gave a *p*-level of 0.49, which verifies that the experimental and calculated p*I* values are not significantly different.

Besides showing that p*I* values for denatured proteins with known compositions can be calculated with a high degree of precision from average p*K* values, the results also provide strong support for the notion that *N*-terminal blockage heavily depends on the nature of the *N*-terminal groups [26]. The results seem to indicate that with *N*-terminals other than M, S and A, only a few proteins have blocked *N*-terminals (1 out of 10 proteins in the present study), while it can be inferred from the data presented in Table 2 that a majority of the proteins with M, S and A as *N*-terminal are blocked. After correction for the effect of suspected *N*-terminal blockage there is only one protein (nucleolar protein B23) out of the 36 used in this study, which, in spite of a high buffer capacity, has a marked difference of 0.11 pH units between predicted and determined p*I* values (Fig. 4B); this corresponds to 3 charge units due to the high buffer capacity of this protein. This discrepancy in p*I* prediction and calculation of net charge at the p*I* is probably not due to deficiencies in the database information but instead reflects a shortcoming of the model used for p*I* calculations. Nucleolar protein B23 contains a domain extremely rich in aspartic and glutamic acid residues (Table 4), in which 26 out of 28 amino acid residues from position 161 to 188 are either a D or an E. A calculation based on the use of average p*K* values uninfluenced by the charged neighboring amino acid residues cannot be expected to correctly describe the p*I* value with almost half of the acidic groups packed together into a highly negatively charged region. This limitation caused by calculations based on average p*K* values does not severely limit the usefulness of the approach since a search through Swiss-Prot shows that this type of D/E-rich motif is uncommon, and the existence of a highly charged region is immediately apparent upon inspection of the amino acid sequence.

Table 4. Amino acid sequence of nucleolar phosphoprotein B23



The quality of the information available in databases, especially concerning posttranslational modifications, is a major problem when the data is to be used for p*I* predictions. The *p*-level of 0.043 found for all 36 proteins after correction for *N*-acetylation, shows that this problem is not only limited to *N*-terminal blockage and the very good agreement found for the eighteen polypeptides, with assumingly correctly described *N*-terminal (Fig. 4C), must be regarded as an exception from this point of view. *N*-Terminal blockage is generally the main problem in relation to p*I* predictions for eukaryotic proteins. Of the 36 keratinocyte proteins analyzed, 18–20 are suspected to be *N*-terminally blocked (6 proteins blocked according to Swiss-Prot, 12 proteins with M, S or A as *N*-terminal and assumingly blocked based on the calculated charge, and two proteins, involucrin and nucleolar protein B23, with M as *N*-terminal for which the data does not allow any conclusion). This is in reasonable agreement with the conclusions based on the *N*-terminal sequencing data derived in connection with 2-D gel electrophoresis. *N*-terminal blockage can be suspected for 17–19 of the 26 proteins with M, S or A as *N*-terminal, while only 1 in 10 proteins with other *N*-terminal groups are blocked. The information that the frequency of *N*-terminal blockage is strongly related to the nature of the *N*-terminal group will be of some help in connection with p*I* predictions based on database information. However, without information from other sources, an uncertainty will always remain as to whether the *N*-terminal charge should be included in the p*I* calculation.

4 Concluding remarks

The data presented here lays the foundation for comparing 2-D gel protein maps of different cell types generated with nonlinear, wide-range IPGs in the first dimension. The focusing positions of 41 polypeptides common to most human cell types have been described in a pH scale that allows focusing positions to be predicted with a high degree of accuracy, provided that the composition of the polypeptides are known and that information on posttranslational modifications are available. For polypeptides with a very high buffer capacity, the limiting factor is the precision with which experimental pH values can be determined rather than the precision of the calculations. Possible deficiencies in the pH scale description of the variation of the hydrogen ion activity has, at least at the present state, no consequences for its practical use. The major limitation in connection with predictions of focusing positions from polypeptide compositions is the quality of existing data on protein compositions, especially concerning posttranslational modifications. Amino acid sequences have been reasonably easy to obtain, while posttranslational modifications

have been difficult and work-intensive to determine. Recent developments in the field of mass spectrometry are fast changing this situation and within the next years we can expect a surge in reliable data in this area. While awaiting this development, verification of correctness and completeness of available information on polypeptide composition can be provided by experimental pI values in a pH scale based on the pI values determined in this study. So far, our data cover the pH range below pH ≈ 7.5. The basic pH range covered by NEPHGE as first dimension will be covered in forthcoming work.

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